Phenylethyl Resorcinol in Niosomes for Cosmetic Formulation

Janejira Buruschat

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Cosmetic Sciences (International Program)

Prince of Songkla University

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ชื่อวิทยานิพนธ์ ฟีนิลเอทิลเรซอซินอลในนิโอโซมสำหรับสูตรตำรับทางเครื่องสำอาง

ผู้เขียน นางสาว เจนจิรา บุรุษชาติ

สาขาวิชา วิทยาศาสตร์เครื่องสำอาง (นานาชาติ)

ปีการศึกษา ๒๕๕๙

บทคัดย่อ

ฟีนิลเอทิเรซอซินอล เป็นสารสังเคราะห์ที่มีฤทธิ์ในการยับยั้งการทำงานของเอนไซม์ไทโรซิเนส ซึ่งเป็นเอนไซม์ที่ทำให้เกิดกระบวนการผลิตเม็ดสีผิวในร่างกายของมนุษย์ ในการทดสอบประสิทธิภาพในการยับยั้งเอนไซม์ไทโรซิเนส โดยเปรียบเทียบกับสาร Kojic acid พบว่าฟีนิลเอทิลเรซอซินอล มีประสิทธิภาพในการทำงานได้ดีกว่าหลายเท่า แต่อย่างไรก็ตามปัญหาความคงทนต่อแสง และการละลายน้ำต่ำ ซึ่งส่งผลต่อการแสดงประสิทธิภาพของสาร ทั้งนี้ยังพบรายงานการระคายเคืองต่อผิว ในผลิตภัณฑ์ที่ใช้กับผิวหน้า จึงนำไปสู่การนำนิโอโซมมาใช้เพื่อปรับปรุงประสิทธิภาพของสาร

ในการศึกษาครั้งนี้จึงได้พัฒนาตำรับของนิโอโซมโดยการเตรียมด้วยวิธี reverse phase evaporation โดยมีความเข้มข้นของสารฟีนิลเอทิลเรซอซินอลในนิโอโซมเท่ากับ 25 มิลลิกรัมต่อมิลลิลิตร พบว่าตำรับที่เหมาะสมที่สุดนั้นประกอบด้วย Brij™72:CHOL ในอัตราส่วน 1:1 โดยโมล และมีความเข้มข้นรวมกันที่ 0.14 มิลลิโมลต่อมิลลิลิตร ซึ่งมีขนาดอนุภาคเท่ากับ 413.00 ± 121.26 นาโนเมตร และมีความสามารถในการกักเก็บสารเท่ากับ 73.50 ± 0.04% ผลการศึกษาความคงตัวพบว่านิโอโซมตำรับนี้มีความคงตัวที่อุณหภูมิห้องเป็นเวลา 6 สัปดาห์ และผลการศึกษาการซึมผ่านในผิวหนังนอกกายหลังจากเวลา 24 ชั่วโมงพบว่ามีการสะสมของสารฟีนิล

เอทิลเรซอซินอลในผิวหนังในปริมาณ 132.40 ± 2.93 ไมโครกรัมต่อตารางเซนติเมตร และมีค่าการซึมผ่านผิวหนังของนิโอโซมเท่ากับ 16.49 ± 1.14%

ในการพัฒนาร่วมกับตำรับทางเครื่องสำอางพบว่า ตำรับที่ประกอบด้วยฟีนิลเอทิลเรซอซินอลนิโอโซมยังคงปริมาณของสารสำคัญมากกว่าตำรับที่ประกอบด้วยสารละลายฟีนิลเอทิลเรซอซินอลเมื่อถูกเก็บไว้ที่อุณหภูมิห้องเป็นเวลา 2 เดือน (73.62 ± 0.93 และ 64.18 ± 1.27% ตามลำดับ) และในการศึกษาการซึมผ่านผิวหนังนอกกายพบว่า ในผิวหนังมีปริมาณของสารฟีนิลเอทิลเรซอซินอลจากตำรับนิโอโซมครีมมากกว่าตำรับที่ประกอบด้วยครีมของสารฟีนิลเอทิลเรซอซินอล (123.32 ± 2.18 และ 56.83 ± 2.25 ไมโครกรัมต่อกรัม) ซึ่งจากการทดลองแสดงให้เห็นว่าการนำนิโอโซมมาห่อหุ้มสารฟีนิลเอทิลเรซอซินอลนั้นสามารถช่วยเพิ่มประสิทธิภาพความคงทนต่อสภาวะภายนอกและเพิ่มการนำส่งและสะสมในผิวหนังได้มากกว่าในรูปของสารละลาย ซึ่งเหมาะแก่การนำมาพัฒนาและใช้ร่วมกับตำรับทางเครื่องสำอาง

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ABSTRACT

Phenylethyl resorcinol (PR) is a synthetic compound that it has anti-tyrosinase property, which tyrosinase is an enzyme substrate for melanin production. In the anti-tyrosinase assay, PR performed higher effective than kojic acid. However, there are many problems such as light stability, poor water soluble and reported of irritation, these problems lead to developing the efficacy of PR by using niosomes.

In this study, niosomes formulations were prepared by reverse phase evaporation method with the same concentration of 25 mg/ml. The suitable formulation was composed of Brij™72 and CHOL at 1:1 molar ratio of 0.14 mmol/ml as total content. It gave 413.00 ± 121.26 nm of particle size and the entrapment efficiency was 73.50 ± 0.04%. PR niosomes showed stability at room temperature for 6 weeks and the result from *in vitro* skin permeation study showed the accumulation of PR in skin after 24 hours was 132.40 ± 2.93 μg/cm^2 and the cumulative amount of PR in receptor fluid was 16.49 ± 1.14%.

The development with cosmetic formulation, PR-niosomes cream showed higher amount of PR than PR cream for 2 months (73.62 ± 0.93 and 64.18 ± 1.27% when storage at room temperature, respectively) and *in vitro* skin permeation

study showed the accumulation of PR from niosomes cream was higher than PR cream (123.32 ± 2.18 and 56.83 ± 2.25 µg/g, respectively). These results showed the efficiency of niosomes for protecting PR from environment and increase the permeation of PR across skin than PR cream, which it suitable for using in the development of cosmetic formulation.

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Janejira Buruschat

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LIST OF ABBREVIATIONS AND SYMBOLS

et. al.	et alli, and other
°C	Degree Celsius
CHOL	Cholesterol
cm^2	Square centimeter (s)
DHI	Dihydroxyindole
DHICA	Dihydroxyindole carboxylic acid
DOPA	Dihydroxyphenylalanine
g	gram (s)
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatrography
HQ	Hydroquinone
J_{ss}	Permeation rate at steady state
K_p	Permeation coefficient
l	litre (s)
LOD	Limit of detection
LOQ	Limit of quantitation
µg	microgram
mg	milligram (s)
MITF	Microphtalmia-associated transcription factor
ml	milliliter (s)

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

mmol	millimole (s)
MSH	Melanocyte stimulating hormone
mV	milli volte (s)
No.	number
%	percent
PBS	Phosphate buffer saline
PDI	Polydispersity index
PR	Phenylethyl Resorcinol
Q_t	Cumulative drug permeated
R^2	Correlation of determination
rpm	Round per minute
RSD	Relative standard deviation
SD	Standard deviation
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
UV	Ultra Violet

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Nowadays, beauty trend mentioned on skin whitening, many people try to use products which claimed whitening or brightening to skin. Human skin exists in a wide range of different colors and gradations, ranging from white to brown to black. This is due to the presence of a chemical and stable pigment known as melanin. Melanin is produced deep inside of the skin and plays an important role in defending the body against harmful UV rays and other environmental challenges (Costin *et al*., 2007). In the past, hydroquinone was used as skin lightening agent in cosmetic products. Hydroquinone interact with the active site of tyrosinase affect the reduction of skin pigmentation, however, it was banned by European Committee (24th Dir 2000/6/EC) because of side effects such as permanent depigmentation of skin and exogenous ochronosis (Gillbro *et al*., 2011). Kojic acid, arbutin, and licorice extract are famous agents used for whitening products in the present. They have the same activity is tyrosinase inhibition. For this study, the novel ingredient was used which it called phenylethyl resorcinol (PR).

Phenylethyl resorcinol (4-(1-Phenylethyl) 1,3-Benzenediol), PR) also known as lightening and brightening ingredient in cosmetic. PR has form in white crystal solid with very low odor. It is a synthetic compound, that can be inhibited tyrosinase activity as well as it can give antioxidant effect (Fan *et al*., 2014). PR is one of

synthetic analog form that has higher activity of whitening agent when compared with kojic acid (Kott *et al*., 2013).

Niosomes are vesicles composed of a non-ionic surfactant with the addition to cholesterol (CHO) or its derivatives. Niosomes can be encapsulated both hydrophilic and lipophilic substance. The hydrophilic substance will locate in aqueous core, and lipophilic will located in the lipid bilayer (Moghassemi *et al*., 2014). Niosomes production was firstly used in cosmetic industrial and then potential application in drug delivery. Poor water solubility of a drug will be increased because property to enhance the solubility of non-ionic surfactant of niosomes. (Mahale *et al*., 2012). Niosomes are one of the best carriers due to the stability of vesicle. Both chemical stability and economy provided niosomes becomes the first role in drug delivery system (Prabhjot *et al*., 2014).

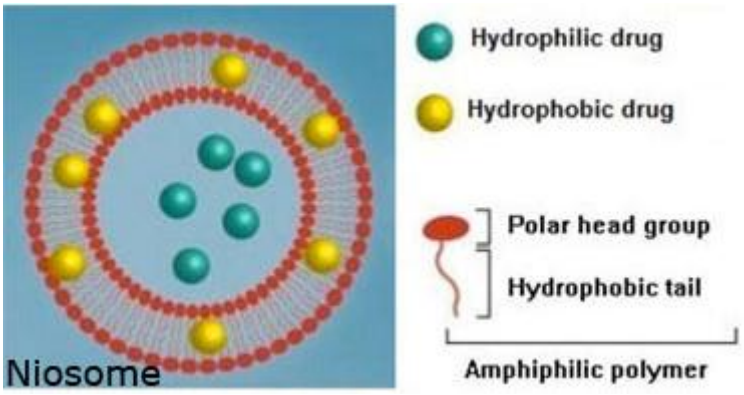


Figure 1.1 Structures of Niosomes.

(Source: http://www.naturalislife.com/niosome.html)

Niosomes has numerous advantages. They have a chemical stable and have long term storage time than liposomes. Their surface formation and modification are very easy because of the functional groups on their hydrophilic heads. They have high compatibility with a biological system and low toxicity because of their non-ionic nature. Niosomes also can enhance absorption of some drug across the cell membrane to localize in target tissues (Moghassemi *et al*., 2014).

Niosomes have been used first in the cosmetic industry and then have come to use in pharmaceutical industry. They have enormous potential for therapeutic applications. Niosomes can encapsulate various drugs such as antioxidant, anti-bacterial and anti-inflammatory.

Depending on the types of drugs, surfactant, disease or location of defects, various routes of administration exist for niosomal drugs, some of the administration routes, however, several routes of administration have been reported. (Moghassemi *et al*., 2014).

From my background and rationale can be concluded that PR was used for cosmetic formulation as lightening ingredient, however, their problem properties reported such as irritation and solubility. Then this study will be used niosomes for solving those problems.

1.2 Objectives

The objectives of the present study were;

1.2.1 To develop niosomes vesicle for encapsulate phenylethyl resorcinol.

1.2.2 To investigate physiochemical property of niosomes containing phenylethyl resorcinol.

1.2.3 To study *in-vitro* skin prenetration of phenylethyl resorcinol niosomes and phenylethyl resorcinol cream and phenylethyl resorcinol niosome cream.

1.2.4 To investigate stability of phenylethyl resorcinol niosomes in cosmetic formulation.

1.2.5 To reduce irritation of phenylethyl resorcinol in cosmetic formulation.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Skin pigmentation

Skin is an organ that can be found in all living species. Skin act as an important barrier to protect mechanical, chemical and microbial from an environment. Another one of the important functions is to against UV rays by pigments. Skin can divided into three layers; epidermis, dermis and hypodermis or subcutaneous. Thus, the UV rays can effect to two layers of skin which are epidermis and dermis. Hypodermis located under the dermis, it connects bones and dermis by fatty tissue (Costin *et. al.,* 2007).

Epidermis is the outer layer of skin, thinner and the arrangement is not complicated like the inner layer, the dermis. Keratinocytes was found in the epidermis and contains 3 types of dendritic cells: (1) Melanocytes have function to produce skin pigment, (2) Langerhans cells associated with the immune system, and (3) Merkel cells synergistic active with nerve cells. The epidemis subdivided into four layers; stratum basale, stratum spinosum, stratum granulosum and stratum cormeum. Nonviable and flattened keratinocytes collocated at stratum corneum. In tanned to dark skin people, melanin pigments distributed at this layer effect to enhance the UV protection function. Melanocytes have role to produce skin pigment or melanin,

located in the stratum basale of the epidermis and the matrix of the hair bulb. It produces melanin in melanosomes which is cytoplasmic organelles. Melanosomes varied in size and term of distribution depends on skin type and pigmentation as shown in Figure 2.1 (Costin *et. al.*, 2007; Jablonski, 2004).

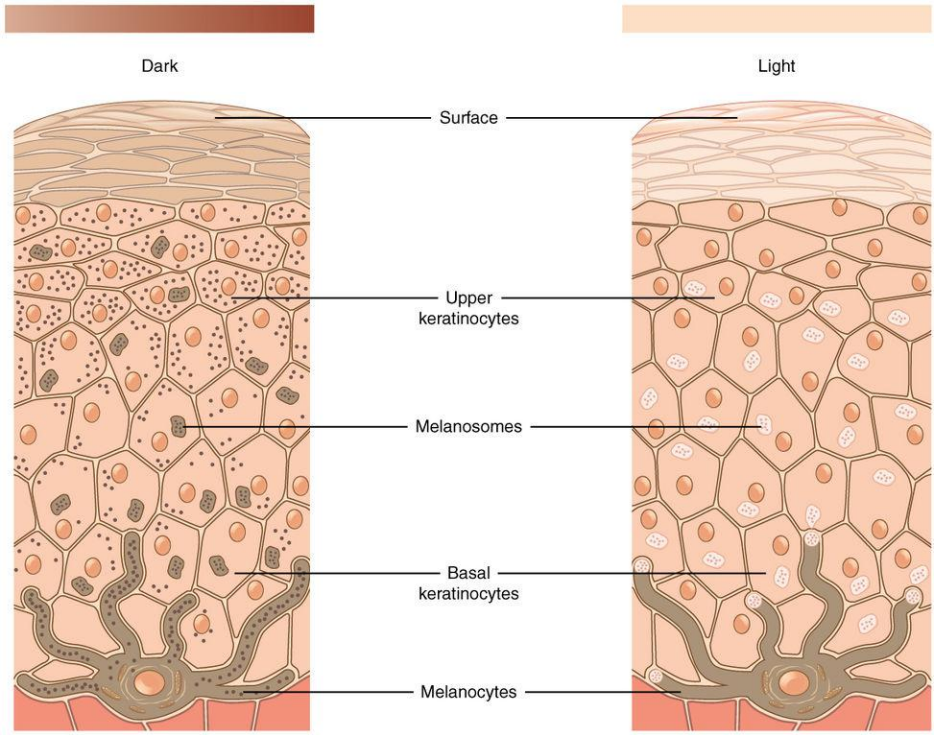


Figure 2.1 The differences of skin pigments (Source: http://commons.wikimedia.org/wiki/File:504_Melanocytes.jpg).

Melanocytes produce two types of melanin, which are eumelanin and pheomelanin. They present different of color, eumelanin present brown to black color and pheomelanin present yellow to red color (Costin *et. al.*, 2007). Mechanism of

melanogenesis start by L-tyrosine was catalyzed by tyrosinase and transformed to dihydroxyphenylalanine (DOPA), after that DOPA was oxidized to DOPAquinone. In the presence of cysteine or glutathione, DOPAquinone will react with them and cystenylDOPA occurred and transform to pheomelanin.

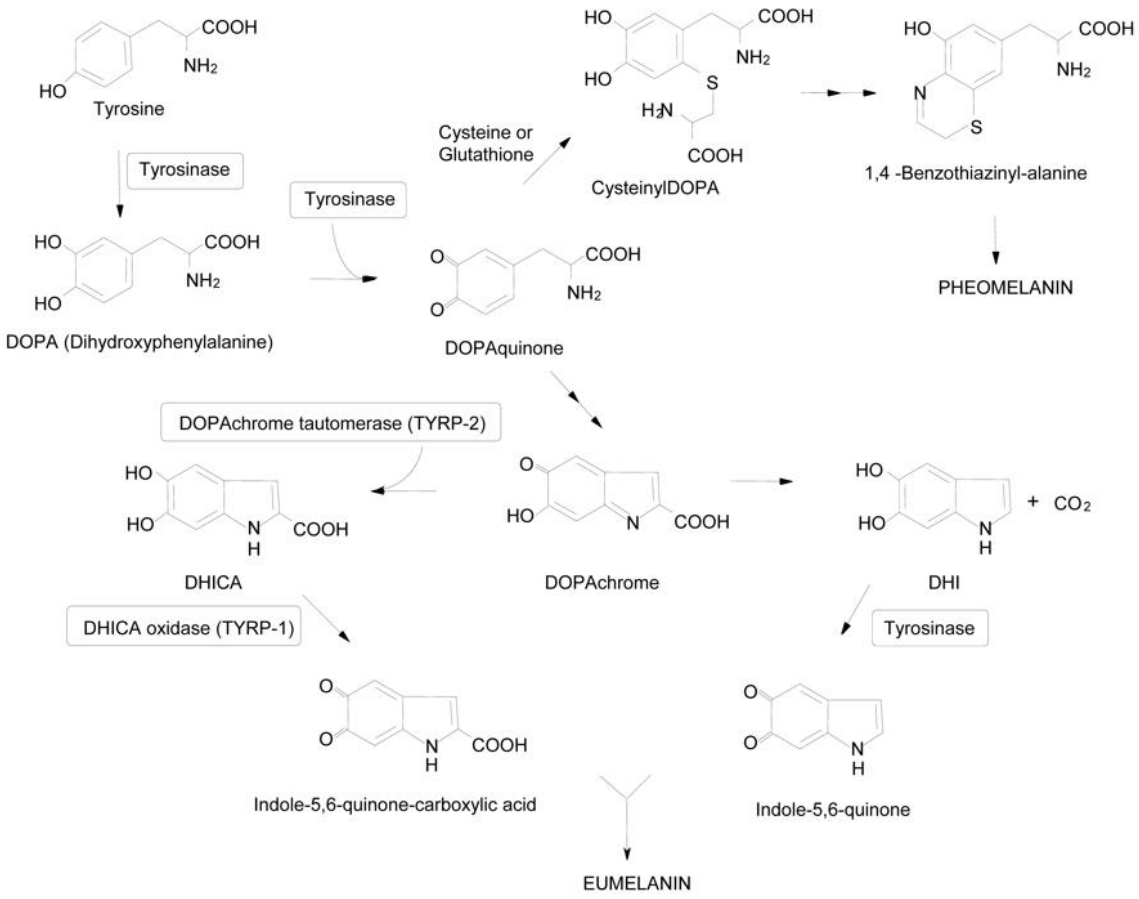


Figure 2.2 Melanogenesis of skin pigments (Ebanks *et. al.*, 2009).

As deplete of cysteine, the cyclization of DOPAquinone to DOPAchrome occurred. After that the tuatomerization occur by DOPAchrome tautomerase (TYRP-2) catalyzes DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Then

it transform to eumelanin by TYRP-1 catalysis. In case of TYRP-2 does not exist, DOPAchrome immediately change to 5,6-dihydroxyindole (DHI) because the carboxylic group lost. Finally, DHICA compose with DHI to form eumelanin (Ebanks *et. al.*, 2009). Melanogenesis occur in melanosomes which are located in melanocyte cell. Melanosomes are transferred to the upper layer of skin through surrounding keratinocytes. Skin color is the outcome of the expression of melanin synthesis and transfer melanosomes to upper skin layer.

The increasing of skin pigmentation can determine by many factors (Figure 2.3). The factors can divide into 2 groups; (1) environmental influences or external factors and (2) endogenous influences or internal factors. Hyperpigmentation of skin by external factors has UV radiation as the key factor. When skin exposure UV, skin defensed by produce thicken stratum corneum and stimulated melanognesis of epidermis. UV rays were scattered and absorbed by keratin and protein in the stratum corneum. Apart from that UV increased melanogenesis and rate of transferring melanosomes to keratinocytes. This action influences tanning skin or another skin problems so solar lentigo, melasma, etc. In addition, using drugs and chemical on the skin can cause hyperpigmentation. Antibiotics, diuretics, nonsteroidal inflammatory drugs stimulated hyperpigmentation skin by increasing melanogenesis and increasing melanocyte cells.

During pregnancy, increasing of hormone level; estrogen, progesterone and MSH affected melanogenosis increasing within melanosome, however, number of melanocyte cell was not increased but it enlarges in size and more dendritic. Another is postinflammatory, many factors and pathways influences are increasing of

tyrosinase activity and production of melanin (Costin *et. al.*, 2007). Hyperpigmentation skin is a result of many reasons such as injury or inflammation, acne or eczema or contact dermatitis that can be found in all skin types.

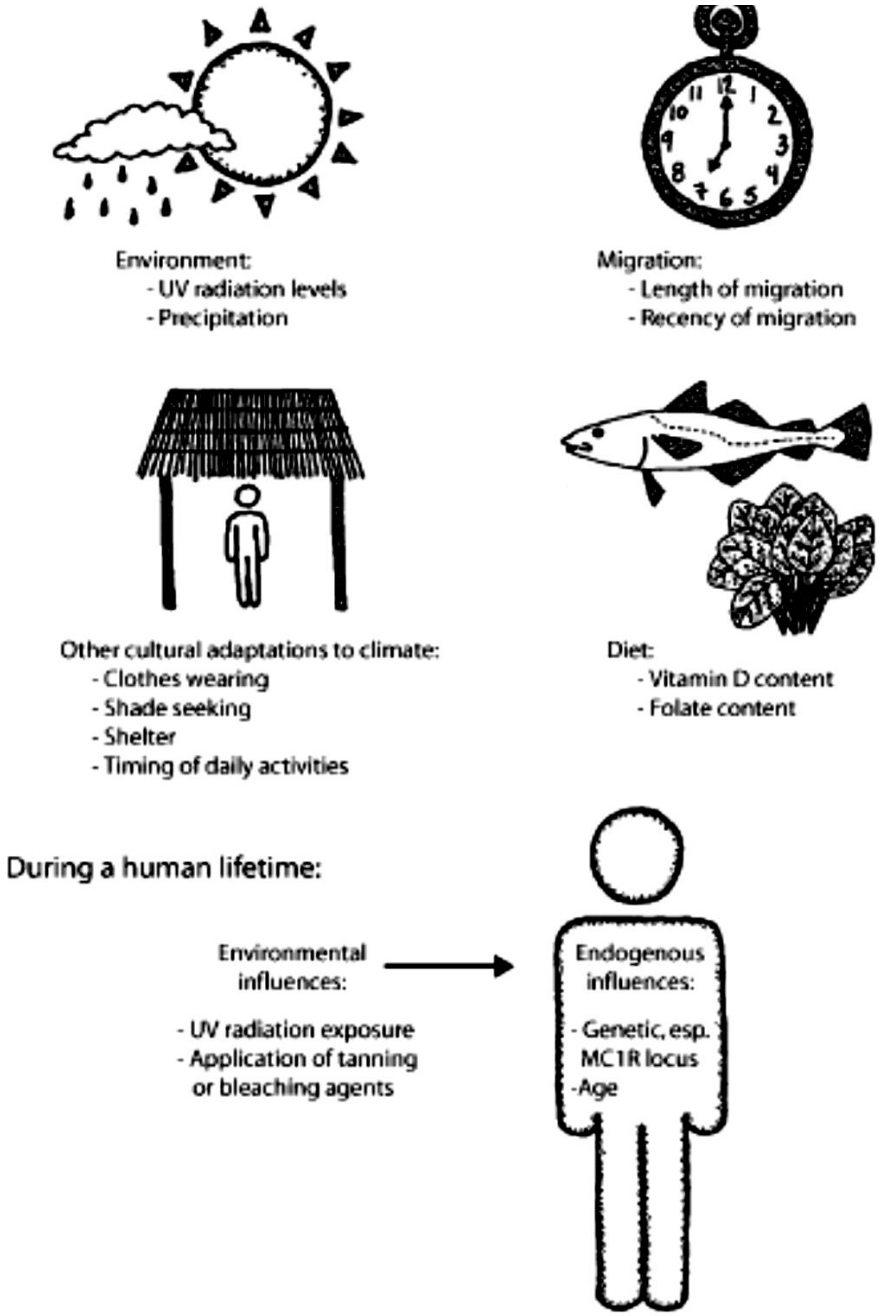


Figure 2.3 The factors influence to pigmentation skin (Jablonski, 2004).

2.2 Skin whitening

In the past decade, tanned skin was infamous in a fashionable for beauty and wealth. Most of women try to get brighten and whiten skin by using cosmetic formulation and avoidance UV rays (Ebanks *et. al.,* 2009; Lin *et. al.,* 2008). Then, using whitening agents with cosmetic formulation becomes popular in the cosmetic industry.

As described above, melanosomes synthesized melanin and they were distributed by melanocytes. Inhibition of melanin synthesis can be reduced amount of melanins which effect to lighten skin. Tyrosinase inhibitor is one of the ways to decrease melanins in systemic. It had been the most common to obtain whitening skin, then almost of whitening agents work by disturbing a mechanism of melanin synthesis (Woolery-Lloyd *et. al.,* 2011). The disturbance can divide into three main groups; (1) before melanin synthesis, (2) during melanin synthesis and (3) after melanin synthesis as shown in Table 2.1.

When skin exposure to UV rays or hormone level changed, the stimulating of melanin synthesis will start. Factors stimulate many chemicals signal result to the increasing of expression of tyrosinase. The microphtalmia-associated transcription factor or MITF is a chemical signal that controls the increasing of melanin pigment, and then degradation of MITF affects the decreasing of melanin production. During glycosylation which is the mechanism of *N*-linked glycan combined with asparagine for tyrosinase producing, the inhibition can occur by inhibit *N*-glycan process this result to decrease tyrosinase activity.

During melanin synthesis tyrosinase enzyme is the major substance in this process, enzyme substrate interaction is the principle of this mechanism. The interruption of tyrosinase to combine with tyrosine is a role of whitening agents in this process. After melanin synthesis, melanosomes was transferred by dendritic ends of melanocytes to keratinocyte cells. The inhibition of melanosomes movement is the role of whitening agents (Son *et. al.,* 2013).

Whitening agents derived from natural and synthetic sources, both of them widely used in the cosmetic industry. In the past, hydroquinone (HQ) widely used in cosmetic products, it works by binding with the active site of enzyme result to inhibit pigmentation. Although, HQ successfully used as whitening agent, but it can be caused side effect known as permanent depigmentation. The European Committee (24^{th} Dir 2000/6/EC) had been banned HQ and withdrawn cosmetic formulations which contained it, but it can use with prescription. Many agents produced for using as whitening agents; (1) arbutin is a botanical derived from fresh fruits. With the structural similarly to HQ, arbutin inhibited pigmentation by binding with active site of enzyme. (2) Kojic acid is one of whitening agent that commonly used in many formulations. Copper atoms quenching and suppressing tautomerization of dopachrome to DHICA are the inactivation of tyrosinase by kojic. (3) Licorice extract is a plant extracted, glabidin is the active component in this extract and performs inhibit tyrosinase activity (Ebanks *et. al.,* 2009). These are commonly used of whitening agent in cosmetic industry. Nowadays have many developed whitening agents launched in the market.

Table 2.1 The disturbing of melanin synthesis in three groups (Son *et. al.*, 2013).

Stage of melanin synthesis	**Mode of action**	**Compounds**
Before melanin synthesis	Tyrosinase transcription	C2-ceramide, tretinoin
	Tyrosinase glycosylation	$PaSSO_3Ca$
During melanin synthesis	Tyrosinase inhibition	Hydroquinone, Kojic acid, Azelaic acid, Resvertrol, etc.
	Peroxidase inhibition	Phenols, Cathecol
	Product reduction and ROS scavenging	Ascorbic acid, α-tocopherol
After melanin synthesis	Tyrosinase degradation	α-Linoleic acid
	Inhibition of melanosome transfer	Niacinamide, Soybean/milk extract
	Skin turn over acceleration	Lactic acid, Glycolic acid

2.3 Phenylethyl Resorcinol

Phenylethyl Resorcinol (PR) is a synthetic compound that adapted from pinosylvin which is the first substance form pine tree, it was unstable form and was hydrogenated to dihydropinosylvin. Dihydropinosylvin was stable form however it difficult to synthesized in this form. Then synthetic dihydropinosylvin analogue was occurred and that is PR (Kott *et. al.*, 2013).

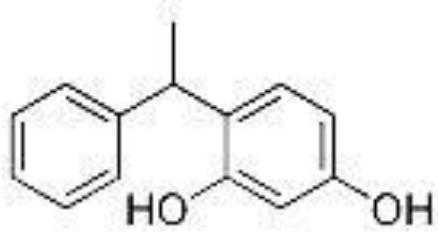


Figure 2.4 Chemical structure of 4-(1-Phenylethyl) 1, 3-Benzenediol.

2.3.1 Physiochemical properties of PR

PR has IUPAC name is 4-(1-Phenylethyl) - 1,3-benzenediol, it is a raw material in cosmetic formulation. Physicochemical properties were shown as below;

Generic name: Phenylethyl Resorcinol

Chemical name: 4-(1-Phenylethyl) - 1,3-benzenediol

Chemical formula: $C_{14}H_{14}O_2$

Molecular weight: 214.27 g/mol

Appearance:	White to beige color powder
Solubility:	3.85 g/l (Method: OECD Test Guideline 103)
Melting point:	78 °C
Log P:	2.11

In addition to Fan *et. al*. (2014) reported that the property of PR about light instability and poor water solubility. The poor water solubility may effect to limit the absorption ability when used. And they developed nanostructured lipid carrier to improve about those problems.

2.3.2 Properties of PR

PR was tested by mushroom tyrosinase inhibition, the test performed by mix mushroom tyrosinase with phosphate buffer (pH 6.8) and test compounds then they were incubated at 37 °C for 10 min and add L-dopa after that quantified by photometric method and determined the inhibition efficiency and the result showed inhibition activity more than kojic acid around 22 times. A second test is epidermal model. Skin type IV models were used and applied to test compounds. Then melanin cell was extracted and determine by photometric method. The results exhibited kojic acid 1% can inhibit melanin synthesis around 80% however PR 0.1% inhibit melanin synthesis around 95% then the efficacy of PR more effective than kojic acid around ten times (Kott *et al*., 2013).

After they got the results in epidermal model test, to ensure the PR was not cause cytotoxic to cell. MTT assay was done by exposed 0.1% PR and incubate for 7

and 14 days. Result exhibited cell still 100% viable. That means PR is not cause cytotoxicity. The others *in-vitro* testing also found the same result which PR effective than kojic acid (Kott *et al*., 2013).

However, Gohara *et al*. (2013), reported that a 52 years old female had erythema appeared after application of a skin lightening essence, composed PR, for reduce hyperpigmentation on her face. Then, skin irritation was tested by patch testing using Finn Chambers® at the upper back for 2 days. Results were reported in accordance of the International Contact Dermatitis Research Group recommendations.

2.4 Niosomes

Niosomes are one of drug delivery system which it composed non-ionic surfactant and derivatives such as cholesterol. Niosomes can be formed by self-assembly in hydration media (Moghassemi *et. al.*, 2014; Azeem *et. al.*, 2009). L'Oreal is the first company used niosome in cosmetic formulation because the aspect of the irritation effect of surfactant which is a major component in the system (Azeem *et. al.*, 2009; Mahale *et. al.*, 2012).

2.4.1 Formulation components

The components are the most important in formulation affect to the characteristic of niosomes. Non-ionic surfactant is principle component in formulation.

2.4.1.1 Non-ionic surfactant

Non-ionic surfactant is polymeric surfactant widely used to regulate, retain, and delivery drug to target sites. Commonly, surfactants have net charges on their polar head group, non-ionic surfactant has no charge at the polar head. Non-ionic surfactant can be classified to various types such as glycerol and glycerol esters, Sorbitan esters, Polysorbates and Macrogol ethers. Using of non-ionic surfactant and drawbacks of them showed in Table 2.2.

2.4.1.2 Membrane additives

Cholesterol

Cholesterol is the most common additive that influences entrapment efficiency, storage time, release and stability of niosomes. At the difference HLB of

surfactant, cholesterol must use to form the bilayer of vesicles. Cholesterol provides gel liquid transition temperature of surfactant that enhances stability of vesicles. In the point of drug entrapment efficiency, cholesterol must use in the optimizing ratio between surfactant and cholesterol, it result to increase entrapment efficiency too.

Charge inducers

Instability of niosomes can induce aggregation of vesicles. Then, the addition of surface charge had been used and it improves stabilization of vesicles. Dicetyl phosphate, stearylamine and diacylglycerol are commonly additive was used as charge inducer.

Table 2.2 Using of non-ionic surfactant and drawback (Mahale *et al*., 2012).

Nonionic surfactant type	**Physicochemical properties**	**Preferable use**	**Drawback**
Alkyl ethers and alkyl glyceryl ethers	High stability	To encapsulate proteins and peptides	Decrease entrapment efficiency when used with cholesterol
Polyoxyethylene 4 lauryl ether (Brij 30)	HLB 9.7 T_c 10°C	Forms large unilamellar vesicles	Incompatible with iodide, mercury salts, tannins sulphonamides and some drugs
Polyoxyehtylene acetyl ethers (Brij 58)	Ability to form invert vesicles due to its large head group (E20-23)	Useful for studying ion-pumping activity at the plasma membrane.	-
Polyoxyethylene fatty acid esters (Brij 72)	Ability to forms multi-lamellar vesicles with high encapsulation efficiency because of low HLB value of 4.7	For vesicle formation having a high encapsulation efficiency	-

Table 2.2 Using of non-ionic surfactant and drawback (Mahale *et al*., 2012) (Continue).

Nonionic surfactant type	**Physicochemical properties**	**Preferable use**	**Drawback**
Sorbitan fatty acid esters	Vesicles made with these higher molecular weight Spans are less leaky and more stable to osmotic gradients.	In cosmetics as solubilizer of essential oil in water-based product	The molar ratio of cholesterol to Span may affect the entrapment of drugs into niosomes.
Gemini surfactants	Lower CMC value, non-toxic, more stable, non-irritating non hemolytic	Personal care and pharmaceutical formulations	-
Bola Surfactants	Higher solubility, higher CMC, lower aggregation number	Personal care and pharmaceutical formulations	-
Ploysorbate	Stabilizer, lower CMC, good water solubility	Use in injections, vaccines	Harmful to persons with Crohn's disease

2.4.2 Advantages and disadvantages of niosomes (Moghassemi *et. al.*, 2014; Azeem *et. al.*, 2012)

Niosomes was developed to deliver drug target site, the advantages of niosomes was summarized as below;

(a) Niosomes has osmotically active and stable.

(b) Because of the hydrophilic head of surfactant, then they can improve surface charge easily.

(c) The nature of non-ionic surfactant result to high compatibility with biological systems and enhance skin permeation of drug.

(d) Niosomes can entrap both hydrophilic and hydrophobic drug.

The instability of niosomes may occur as liposomes in during of dispersion of vesicles. Aggregation, fusion,and leakage of drug are the risk of instability.

2.4.3 Types of niosomes

Niosomes can be classified into many types due to the mention of study. In common, niosomes were classified by size of vesicles which can divide to three types. Firstly is small unilamellar vesicles (SUV) that has size 10 to 100 nm. Second is large unilamellar vesicles (LUV) has size 100 to 3000 nm. The last is multi-lamellar vesicles (MUV) that has more than one bilayer, 1000 nm in size.

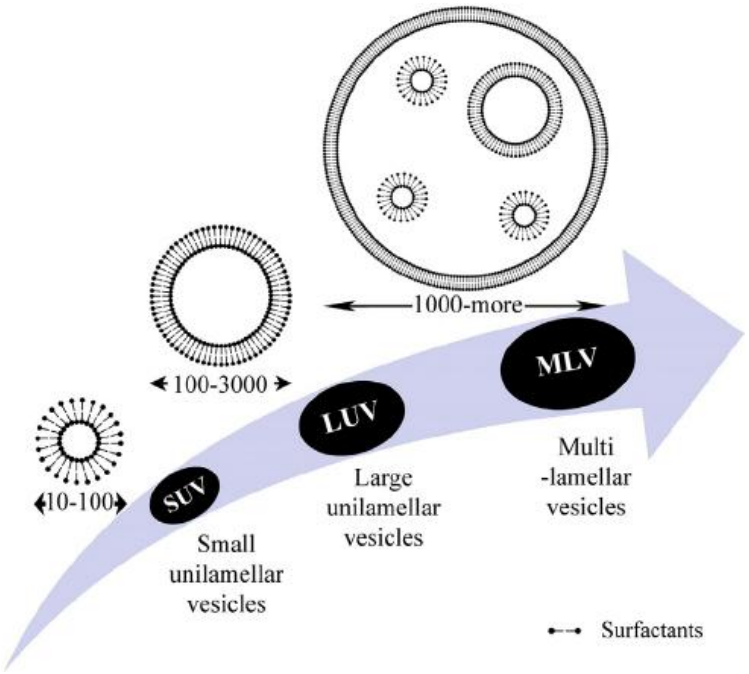


Figure 2.5 Niosomes types classified by size of vesicles (Mahale *et.al.*, 2012).

2.4.4 Methods of preparations (Moghassemi *et. al.*, 2014; Azeem *et. al.*, 2009; Mahale *et. al.*, 2012)

Preparation methods of niosomes base on the hydration of surfactant by using hydration medium. Many methods are shown as following.

2.4.4.1 Ether injection

The surfactant and additive are dissolved in hydration medium and inject through a needle into aqueous phase which contained drug and constant heated. The organic solvent is evaporated by rotary evaporator, the formation of vesicle are formed in this step. LUVs are almost size of vesicle of this method and give high entrapment efficiency.

2.4.4.2 Thin film hydration

The thin film hydration method is widely used. Surfactant and additive are dissolved by organic solvent, and thin film occurs after remove organic solvent by

rotary evaporator. After that aqueous solution is added to hydrate the film, vesicles form in this step. The result of this method provided MLVs with low entrapment efficiency.

2.4.4.3 Reverse phase evaporation

The surfactant with additive are dissolved by organic solution, aqueous phase with drug is added. Then the mixture is sonicated and evaporated organic solvent result to gel form.

2.4.4.4 Trans membrane pH gradient method

This method is prepared by dissolving surfactant and additive with organic solvent and evaporates to get thin film, then hydration film by citric acid solution and freeze/thaw it. After that, aqueous phase is added and vortexed. The pH is raised to 7.0-7.2 by disodium hydrogen phosphate.

2.4.4.5 Sonication method

An aqueous phase of drug is mixed with surfactant/additive mixture. The mixture is sonicated for a certain time. The result is SUVs and less entrapment efficiency.

2.4.4.6 Microfluidization

The method gives uniformity of vesicles size distribution, and based on submerged jet principle which the solution of surfactants and drug is pumped through the desired size of channels.

2.4.4.7 Bubble method

This method can prepare niosomes without using organic solvent. The glass reactor with three necks was used in the process, first neck contained thermometer, the second is nitrogen and the last is water-cooled reflux. Surfactant,

additive, and drug are dispersed at 70°C and followed by the bubbling method of nitrogen gas.

2.4.5 Characterization (Moghassemi *et. al.,* 2014; Mahale *et. al.,* 2012)

2.4.5.1 Morphology

In commonly, niosome is assumed to be spherical in shape. Various techniques can be determined such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

2.4.5.2 Vesicle size, zeta potential, and polydisperity index

The zeta potential of vesicles performed the behavior of niosomes which affect to distribution of system against aggregation or fusion of vsicles.

2.4.5.3 Stability study

Stability of niosomes performed the feasibility of the storage of development formulation. It influences entrapment efficiency of vesicles. Many factors effect to stability such as type of surfactant, cholesterol content, formulation concentration, etc.

2.4.5.4 Entrapment efficiency

The entrapment efficiency is an important effect of niosomes. After preparation, unencapsulate drug is determined by dialysis, uncentrifuge or filtration, and total drug is determind by spectrophotometer instrument, high-performnace liquid chromatography (HPLC) and etc. The entrapment efficiency is determined from concentration which obtained from calculation by using following eqation;

$$\% \text{ Entrapment efficiency (\%EE)} = \frac{\text{Total drug amount - free drug amount}}{\text{Total drug amount}} \times 100$$

2.4.6 Applications of niosomes

Niosomes are first used in cosmetic industry, because of their advantages they are developed to use in pharmaceutical therapeutic. Niosomes can entrap widely drugs and can use with various applications such as anti-oxidants, anti-inflammation and the others as shown in Table 2.3. In addition, niosomes also applied to many administration routes as indicated in Figure 2.6 (Mahale *et. al.*, 2012).

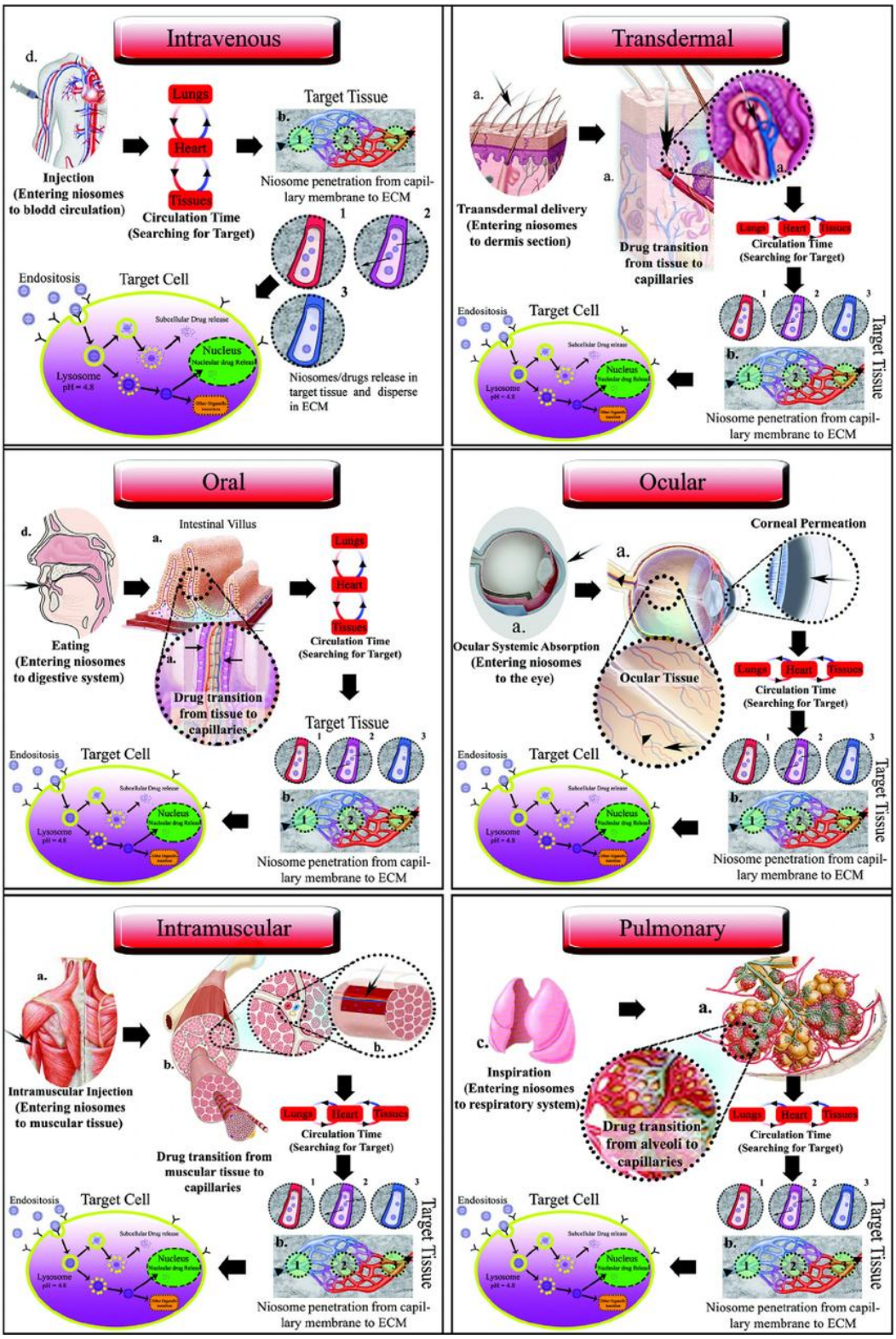


Figure 2.6 Routes of administration of niosomes (Mahale *et. al.*, 2012).

Table 2.3 Application of niosomes for drug delivery (Mahale *et. al.*, 2012).

Loaded drug	**%EE**	**Type of surfactant**	**Preparation method**	**Route of administration**	**Application**
pCMSEGFP	No data	Combination of a cationic lipid, Tween 80 and squalene	REV	Ocular	Gene delivery to retina
Antioxidant (gallic acid, ascorbic acid)	59.40	Tween 60	TFH	Oral	Nutraceutical applications, prevent diseases caused by oxidative strees
Beclometasone dipropionate (BDP)	27.53	Span 60	TFH	Pulmonary	Anti-asthma drug
Morin hydrate	98.62	Span 60, 80 and Tween 60	HSM	Intra-venous	Antioxidant and anticancer drug

Table 2.3 Application of niosomes for drug delivery (continued) (Mahale *et. al.*, 2012).

Loaded drug	**%EE**	**Type of surfactant**	**Preparation method**	**Route of administration**	**Application**
Hydroxychloroquine (HQ)	86.40	Tween 20	REV, EIM, HSM, sonication	Oral	Antimalarial drug and improve oral lichen planus
Ellagic acid	38.73	Span 60 and Tween 60	REV	Transdermal	Antioxidant drug
Diallyl disulfide (DADS)	74.5	Span 20,40, 60, 80	Sonication	Intraperitoneal	Anti-bacterial and anti-fungal drug

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

1. Absolute ethanol (Labscan Asia Co., Ltd, Bangkok, Thailand)
2. Acetonitrile (Labscan Asia Co., Ltd, Bangkok, Thailand)
4. Cholesterol from lanolin (Fluka, Buchs, Switzerland)
5. Carbopol® ultrez 21 (High Science Limited Partnership, Songkhla, Thailand)
6. Disodium EDTA (Namsiang International Co., Ltd, Bangkok, Thailand)
7. Disodium hydrogen orthophosphate anhydrous (Univar®, New South Wales, Australia)
8. Ethanol 95% (Labscan Asia Co., Ltd, Bangkok, Thailand)
9. Glycerin (P.C. Drug Center Co., Ltd, Bangkok, Thailand)
10. Isopropyl Myristate (P.C. Drug Center Co., Ltd, Bangkok, Thailand)
11. Methanol (Labscan Asia Co., Ltd, Bangkok, Thailand)
12. Polyoxyethylene (2) stearyl ether, Brij™72 (J.R. Serve Co., Ltd., Bangkok, Thailand)
13. Polyoxyethylene (80) sorbitan monooleate, Tween 80® (P.C. Drug Center Co., Ltd, Bangkok, Thailand)

14. Propylene glycol (P.C. Drug Center Co., Ltd, Bangkok, Thailand)

15. Sodium cholide (Carlo Erba, Milan, Italy)

16. Sodium dihydrogen orthophosphate (Univar®, New South Wales, Australia)

17. Sodium hydroxide (Merck, Darmstadt, Germany)

18. Tocopherol acetate (P.C. Drug Center Co., Ltd, Bangkok, Thailand)

19. Uniphen P-23 (P.C. Drug Center Co., Ltd, Bangkok, Thailand)

3.2 Instrumentations

1. Electrical balance (AB135-S, Mettler Toledo, Switzerland)

2. High Performance Liquid Chromatography (Agilent Technologies Deutschland GmbH, Agilent 1100 series pumping system, Waldbronn, Germany)

3. Homogenizer (Polytron® system PT 1200 E, Kinematica AG, Littau-Luzern, Switzerland)

4. Hot air oven (DIN 12880-KI, Memmert GmbH, Schwabach, Germany)

5. Magnetic stirrer (MR 3000D, Heidolph, Schwabach, Germany)

6. Modified Franz diffusion apparatus (57-6 M, Hanson Research Corporation, USA)

7. pH meter (UB-10, Denver Instrument, Germany)

8. Refrigerated Centrifuge (KUBOTA 5922 S/N R11419-F000, KUBOTA, Japan)

9. Rotary evaporator (Eyela® N-1000, Tokyo Rikakikai Co., Ltd., Tokyo, Japan)

10. Scanning electron microscope (Quanta 400, FEI, Brno, Czech Republic)

11. Sonicator (HT Crest, S.V. Medico Co., Ltd, USA)

12. Ultracentrifuge (Optima L-100XP, equipped with SW 60 Ti rotor, Beckman Coulter, United States)

13. Viscometer (DV-III ultra, Brookfield Engineering Laboratories Inc, USA)

14. Vortex mixer (G-560E, Scientific Industries Inc., United States)

15. Zeta potential analyzer (ZetaPALS, Brookheaven Instruments Corporation, United States)

3.3 Methods

3.3.1 Preparation of niosomes formulations

Niosomes were prepared by using reverse phase evaporation technique which has been reported about an efficiency method because it gave high entrapment efficiency and high percent release ability of niosomes (Bendas *et al*., 2013). The compositions of niosomes were shown in Table 3.1.

Ingredients Non-ionic surfactant: Brij™72 (Polyoxyethylene (2) stearyl ether)

Additive: Cholesterol (CHOL)

The preparation was prepared by dissolving Brij™72, CHOL and PR in absolute ethanol and sonicate at 45°C for 15 minutes, all components were completely dissolved. Then, the warm water was added to the mixture and sonicating again for 15 min. The mixture was transferred to round bottom flask in order to evaporate organic solvent by rotary evaporator under reduced pressure at 60°C. The suspension was kept in closed bottle glass for further study.

These formulations were prepared and evaluated for physical appearances, particle size, zeta potential and entrapment efficiency for selecting of optimized formulation which it was selected from particle size less than 500 nm, zeta potential more than 30 mV and entrapment efficiency more than 50% for study stability and skin permeation.

Table 3.1 Formulation of PR-niosomes.

No.	Brij™72 (mmol)	Cholesterol (mmol)	PR (mg)	Ethanol (ml)	Water (ml)
1	1.00	0.00	250	10	10
2	0.90	0.10	250	10	10
3	0.80	0.20	250	10	10
4	0.70	0.30	250	10	10
5	0.60	0.40	250	10	10
6	0.50	0.50	250	10	10
7	0.40	0.60	250	10	10
8	0.60	0.60	250	10	10
9	0.70	0.70	250	10	10
10	0.80	0.80	250	10	10
11	0.90	0.90	250	10	10
12	1.00	1.00	250	10	10

3.3.2 Characterization of PR-niosomes

3.3.2.1 Physical appearances

All formulations were observed physical appearance by visual observation on form of color, phase separation and sedimentation.

3.3.2.2 Particle size and Zeta-potential

Zeta potential Analyzer (Zeta PLAS, Brookhaven) was used to determine for characterization of niosomes. Niosomes formulations (100 µl) were

diluted to appropriate volume with distilled water (4 ml) and analyzed at 25°C with 90 degrees of scattering angle. All of them were done in triplicate.

3.3.2.3 Surface morphology of niosomes vesicle by scanning electron microscopy

Niosomes contained PR was examined using scanning electron microscopy (SEM). Firstly, 200 µl of sample was diluted with 3 ml of Milli-Q water. A drop of diluted sample was dried on cover slip and stained with crystal violet solution for 1 min. After that, excess dye was removed by water and fixing with Gram's iodine solution for 1 min. The sample was then coated with gold in a sputter coater under an argon atmosphere (50 Pa) at 50 mA for 50 seconds and investigated under SEM at 80,000X magnification.

3.3.2.4 Entrapment Efficiency

Entrapment efficiency of niosomes was determined by High performance liquid chromatography (HPLC). Niosomes were disrupted by methanol for total active content. A 0.5 ml of niosomes was mixed to 0.5 ml of methanol, and then the mixture was filtrated through 0.45 µm nylon filter before analyzed by HPLC technique. For free active content, a 6 ml of niosomes was pipetted in to centrifuge tube after that it was centrifuged at 4°C, 40,000 rpm for 90 min. A 0.5 ml of supernatant was collected and mixed to 0.5 ml of milli-Q water. The mixture also was filtrated through 0.45 µm nylon filter before analyze by HPLC technique which of the following represents the calculation equation below;

$$\%\text{Entrapment efficiency} = \frac{T - F}{T} \times 100$$

Where; T is total active amount in the formulation

F is the amount of un-entrapped active in the formulation

3.3.3 Stability study of PR niosomes

The selected niosomes formulation was evaluated for stability at 4°C and room temperature for 2 months. The physiochemical properties were evaluated such as physical appearance, particle size, zeta potential and entrapment efficiency as described in section 3.3.2. The evaluations were studied in triplicate.

3.3.4 Skin permeation study

3.3.4.1 Preparation of skin model

The penetration experiment performed by using skin of newborn pigs, it obtained from local farm in Songkhla province. New born pigs that naturally died after birth were cut and cleaned skin surface. After that subcutaneous fat will be removed and skin was cut to appropriated sizes (1.77 cm^2). Skin samples were rinsed with phosphate buffer solution (pH 7.4), wrapped by aluminum foil and kept at -20°C before the experiments (Cilurzo *et al*., 2007). Before beginning of each experiment, the skin was hydrated by immersing in phosphate buffer solution around 2 hours at room temperature.

3.3.4.2 *In vitro* skin permeation study

The selected PR-niosomes formulation was studied *in vitro* skin permeation by compared with PR-solution using modified Franz-diffusion cell. Receptor compartment contained with 11 ml of mixture of 0.44% w/v sodium chloride (NaCl) in phosphate buffer (pH 7.4): propylene glycol (80: 20 % v/v) and stirred with magnetic stirrer at 500 rpm. Skin was mounted on Franz-diffusion cell which has effective area of diffusion around 1.77 cm^2 and it was mounted between donor compartment and receptor compartment. A 1 ml of sample was applied to skin surface on donor compartment. At a period of times (0.5, 1, 2, 4, 6, 8, 12, and 24 hours), receptor fluids were sampling at amount 1 ml and replacing by fresh receptor fluid in the same of amount to maintain a sink condition of experiment. The samples were filtered by nylon membrane and amount of PR will be determined by HPLC method. The samples were analyzed.

3.3.4.3 Determine the amount of PR content in pig skin

At the end of experiments, skins were determined the retain amount of PR in pig skin. The skin was immersed in 5 ml of methanol and cut to small pieces by homogenizer at 24,000 rpm at room temperature for 5 minutes. Next, the samples were centrifuge at 10,000 rpm for 15 minute at 4°C. After that, the sample was filtered through nylon membrane filter before analyzed by HPLC method. The experiment was done in triplicate.

3.3.4.4 Data Analysis

Cumulative amount of PR was calculated the permeation per unit area (Q_t, µg/cm^2) by using the following equation;

$$Q_t = \frac{C_n V + \sum_{i=1}^{n-1} C_i S}{A}$$

Where; Q_t : cumulative amount of PR permeated per unit area of skin (µg/cm^2)

C_n : concentration of PR determined at No. n sampling interval (µg/ml)

C_i : concentration of PR determined at No. I sampling interval (µg/ml)

V : volume of individual Franz diffusion cell (ml)

S : volume of sampling aliquot, 1 ml

A : effective diffusion surface area, 1.77 cm^2

The cumulative amount was plotted as a function of time. In addition, the flux of PR at steady state (J_s µg/cm^2/h) through the pig skin was calculated from slope of linear portion of the plot and permeability coefficient (K_p, cm^2/h) was calculated from following equation;

$$K_p = \frac{J_{ss}}{C_0}$$

Where; C_0 : initial concentration of PR in the donor compartment

3.3.5 Cosmetic formulation

3.3.5.1 Formulation of cream base

Preparation of cream base was performed by beaker method. Ingredients of cream base were showed in Table 3.2 and divided into 2 phases. The ingredients were accurately weighed and separated into two beakers which are phase A and B. Then, they were mixed until homogeneously. After that, phase B was added into phase A and mixed until homogeneously and kept in sealed glass bottle under room temperature. The formulations were evaluated for stability by freeze thaw cycle (1 cycle; 4°C for 24 hours and 45°C for 24 hours) for 5 cycles. Cream bases were studied accelerating stability for selecting formula which has good characteristics such as homogeneous texture, no change in color and odor, pH 4.5-5.5, and viscosity 30,000-50,000 cP. A good formula was incorporated with PR solution and PR-niosome as following experiment.

Table 3.2 Ingredients of cream base.

Phase	Ingredients	Content (g)		
		Formulation 1	**Formulation 2**	**Formulation 3**
A	DI water	To 100	To 100	To 100
	Disodium EDTA	0.10	0.10	0.10
	Carbopol® ultrez 21	0.50	0.60	0.70
	Sodium hydroxide (10% solution)	0.70	0.70	0.70
B	Propylene glycol	2.00	2.00	2.00
	Glycerin	3.00	3.00	3.00
	IPM	5.00	5.00	5.00
	Tween 80	2.00	2.00	2.00
	Tocopherol acetate	2.00	2.00	2.00
	Uniphen P-23	0.50	0.50	0.50
	Fragrance	0.25	0.25	0.25

3.3.5.2 Formulation of cream containing PR niosomes

PR cream and PR-niosome cream were prepared in the same method of cream base. However, PR solution and PR niosomes 20 g was added in cream base (80 g) for total concentration is 0.5% w/w (Table 3.3). The mixture was stirred until homogeneously and kept into glass container at room temperature.

3.3.5.3 Quantitative analysis of PR in the formulations

The content of PR in cream and PR-niosomes in cream were determined by dissolving one gram of cream with 10 mL of methanol, after that the mixture were centrifuged at 10,000 rpm for 30 min at 4°C. Supernatant will be analyzed by HPLC technique (El-Menshawe *et. al*., 2013). The experiment will be performed in triplicate.

3.3.5.4 pH measurement

The formulations were measured pH value using pH meter. The measurement was done in triplicate.

3.3.5.5 Viscosity measurement

Viscosity of formulation was measured at 25°C by using Brookfield DV-III Ultra Rheometer (Brookfield Engineering Laboratories Inc, USA). Brookfield Rheocalc operating software (version3.1-1) controlled the Rheometer. The measurement was performed in triplicated at one point of viscosity.

3.3.6 Stability study of cosmetic formulation

Cosmetic formulations (cream base, PR cream and PR-niosome cream) were studied for accelerated stability by freeze-thawing method. The formulations were stored for 5 cycles (1 cycle; 4±2°C for 24 hours and 45±2°C 24 hours). Furthermore, the stability was studied by storage at 4°C, room temperature (30±2°C) and 45°C for

two months. Every one month, physicochemical properties were analyzed by previous discussed methods. Each experiment was done in triplicate.

Table 3.3 Formulations of cream base contained PR solution and PR-niosome.

Formulations	Cream base (g)	PR solution (g)	PR-niosome (g)	Amount of PR (%)
PR solution cream	80	20	-	0.5
PR-niosome cream	80	-	20	0.5

3.3.7 *In vitro* skin permeation study

3.3.7.1 *In vitro* skin permeation study

The experiment studied *in vitro* skin permeation by comparing between PR cream and PR niosomes cream which the same concentration of PR and cream base. Modified Franz diffusion cell was used in the experiment and it was done as previous describes in section 3.3.4.2. Cream sample was applied on surface of skin in amount of 1 g. The experiment was done in triplicate.

3.3.7.2 *In vitro* skin retention experiment

Determination the amount of PR retained in pig skin was analyzed as previous described in section 3.3.4.3. The experiment was done in triplicate.

3.3.8 Skin irritation

Skin irritation was performed by Thailand institute of scientific and technological research. The experiment was performed following guideline of the test guideline (TG) No. 404: Acute dermal irritation/corrosion test of OECD guidelines for testing of chemical (2002). White rabbits were used in the experiment, rabbits were removed hair on back in area of 10 × 10 cm^2. The gauze patch (size 2.5 × 2.5 cm2) was filled with 0.5 ml of sample and applied on prepared area on back of rabbit covered by adhesive tape for 4 hours, distilled water was used as control. Investigation irritation on skin will be observed at time 1, 24, 48 and 72 hours or more than these, level of erythema and odema on skin will be express as Table 3.4.

Irritation of skin will study by using PR-solution as positive control and distilled water as negative control. PR-niosomes will be study and investigate irritation on rabbit skin. This study will give the result to evaluate the efficiency to reduce irritation of PR by niosomes.

3.3.9 Statistical analysis

All experiment data were represented as mean±SD and were done as triplicate. The content of PR was tested by using linear regression and presented by correlation of determination (R^2). The analyzing of stability testing was performed by the student's t-test analysis with significance level at $p \leq 0.05$.

Table 3.4 Level of erythema and odema formations.

Erythema formation	**Level**	**Oedema formation**	**Level**
No erythema	0	No oedema	0
Very-slight erythema (barely perceptible)	1	Very-slight oedema (barely perceptible)	1
Well-defined erythema	2	Slight oedema (edges of area well-defined by definit raising)	2
Moderate to severe erythema	3	Moderate oedema (Raised approximately 1 mm.)	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4	Severe oedema (raised more than 1 mm. and extending beyond the area of exposure)	4

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Formulation and preparation of niosomes containing PR

The PR-niosomes formulations were prepared by using reverse phase evaporation technique, its compositions and physical characteristics were shown in Table 4.1. The concentration of PR loaded in niosomes formulations was 25 mg/ml (0.5% PR w/w in cream formulation). All of them were characterized by measure vesicle size, zeta potential, and entrapment efficiency. In formulation no. 1-7 had total amount of both surfactant and cholesterol at 1 mmol, these formulations exhibited vesicle size around 240 to 540 nm, zeta potential have range from -33.20 to -40.78 mV and entrapment efficiency around 0.59 to 55.07%. Niosomes formulations were investigated for good formulation (highest entrapment efficiency at 55.07%) and it was improved entrapment efficacy by increasing total lipid content at 1:1 molar ratio between surfactant and cholesterol. In formulation no. 8-12 have total amount both surfactant and cholesterol at 1.2, 1.4, 1.6, 1.8 and 2.0 mmol, its characterizations exhibited around 413 to 1107.53 nm of vesicle size, -29.75 to -42.17 mV of zeta potential and entrapment efficiency around 31.40 to 84.60%. Figure 4.1 shows the physical appearance of PR-niosomes formulations at 1 mmol of total content, which had yellow-orange clear solutions.

Niosomes composed non-ionic surfactant and cholesterol to form vesicle. Brij™72 was used as surfactant in this study. The effect of compositions between

surfactant and cholesterol ratio and total lipid content in formulation had affect to vesicle size and entrapment efficiency (Azeem et. al., 2009). Table 4.1 shows the results of the effect of ratio between surfactant and cholesterol, Figure 4.1 shows the physical appearance of niosomes formulation at 1 mmol. From the left to right side, the formulations were increased cholesterol content 10% in each formulation, respectively. All of formulations were investigated for precipitation or sedimentation which at 1 mmol of total content have similarly in sedimentation time at room temperature, except formulation no. 1 and 2 (Sedimentation in 3 days). After that there are difference times of sedimentation when increased total lipid content, formulation no. 9 has highest stable (6 weeks) of formulation than others. The morphology of niosomes in formulation no. 9 was investigated by using SEM as showed in Figure 4.2. The vesicles formed spherical in shape, and had variation in size.

The effect of cholesterol content on entrapment efficiency and vesicle size of niosomes was studied in many researches. According to result of Essa (2010) and Kamboj *et. al.,* (2014) reported that the increasing of cholesterol content affect to enlarge vesicle size, and size distribution of niosomes. This phenomenon was explained that cholesterol combined with alkyl chain length of surfactant and localized itself between bilayer of vesicles results to increasing size of niosomes (Abdelkader et. al., 2010). Additionally, the increasing of cholesterol content can increase and decrease entrapment efficiency of vesicles, this can explain by conclusion of Mokhtar et. al., (2008) who studied effect of cholesterol on entrapment efficiency by incorporates with Span 20, 40, 60 and 80. They found the different amount of cholesterol affect to entrapment efficiency in difference types of non-ionic

surfactant, and they concluded that it has two conflicts of factors. First is the increasing of cholesterol content influence to increasing of hydrophobicity of bilayers and stability of vesicle and the second is higher amount of cholesterol will localize within bilayer than drug, then drug was excluded from vesicle. In our study, the effect of cholesterol on vesicle size and entrapment efficiency can be described by above. Increasing of cholesterol 10% in each formulation affect to both vesicles size and entrapment efficiency of niosomes. Similarly to Abdelkader *et. al*., (2010) result that increasing of cholesterol affect to increase encapsulate naltrexone hydrochloride (NTX) into niosomes, however, its vesicle size decreased, respectively.

The excess amount of cholesterol can be found in the experiment of Amanatfard *et. al*., (2015) who studied the preparation of niosomes to entrap DEET which they found excess amount of cholesterol as crystal in formulation and size of niosomes also decreased. Then, the optimization of ratio between surfactant and cholesterol was studied, PR-niosome also studied to find optimizing ratio. At 1 mmol of total content with constantly amount of PR (25 mg/ml), PR-niosomes was prepared from 100% of surfactant without cholesterol, then decreasing of surfactant at 10% while increased 10% of cholesterol until 40:60% of molar ratio was found. And results performed highest entrapment efficiency at 1:1 molar ratio which is 55.07%, and the formulation no. 7 (4:6 molar ratio between surfactant and cholesterol) showed white crystal precipitation after evaporated organic solvent. As same as the result of NYS niosomes at 1:1 molar ratio of surfactant and cholesterol also showed highest entrapment efficiency when compared with 2:1 molar ratio (El-Ridy *et. al*., 2011). The effect of surfactant and cholesterol ratio also studied by many researches (Bendas *et. al*., 2013; Moktar *et. al*., 2008; Kamboj *et. al*., 2013), the difference ratio also gave

difference entrapment efficiency because of the increasing cholesterol result to increase membrane rigidity and less leakage of drug, however, at 1:1 molar ratio gave highest entrapment efficiency than others. In addition, the adding of surfactant and cholesterol to 1:1 of weight ratio also increased both stability and entrapment efficiency of RSV niosomes (Pando *et. al*., 2013).

Then PR-niosomes at 1:1 molar ratio was used to improve entrapment efficiency by increasing total content to 1.2, 1.4, 1.6, 1.8 and 2.0 mmol. The results showed highest entrapment efficiency (84.60%) at 1.2 mmol of total content after that entrapment efficiency decreased to 31.40% at 2.0 mmol of total content. As same as the results of salicylic acid (SA) niosomes and benzoic acid by using span 60 and span 80, the entrapment efficiency increased when total lipid concentration was increased from 5.0x10-5 to 2.0x10-4 mol/L. However, the percent entrapment of SA dropped at 4x10-4 mol/L, which it may be occurred from cholesterol taking part of bilayer than drug (Hao and Li, 2011).

Table 4.1 PR-niosomes composition and physical characteristics.

No.	Size (nm)	Zeta (mV)	%Entrapment efficiency	Sedimentation	
				4°C	30°C
1	347.33 ± 15.73	-39.89 ± 1.21	0.59 ± 0.69	3 days	3 days
2	358.20 ± 23.34	-39.96 ± 0.91	2.66 ± 0.18	3 days	3 days
3	544.20 ± 35.72	-40.78 ± 0.63	43.08 ± 0.34	3 days	1 week
4	240.73 ± 14.64	-39.33 ± 1.64	37.53 ± 0.30	3 days	1 week
5	318.35 ± 25.46	-40.16 ± 1.92	20.47 ± 0.42	3 days	1 week
6	362.03 ± 35.23	-33.20 ± 1.75	55.07 ± 0.20	3 days	1 week
7	330.37 ± 73.06	-36.79 ± 1.57	29.28 ± 0.74	3 days	1 week
8	634.93 ± 70.65	-29.75 ± 0.37	84.60 ± 0.07	1 week	2 weeks
9	413.00 ± 121.65	-32.31 ± 0.97	73.50 ± 0.04	1 week	6 weeks
10	503.23 ± 149.74	-30.54 ± 3.08	61.50 ± 0.10	1 week	4 weeks
11	817.20 ± 123.29	-42.17 ± 0.53	76.70 ± 0.05	1 week	4 weeks
12	1107.53 ± 196.45	-33.82 ± 4.65	31.40 ± 0.37	1 week	4 weeks

*Results were represented as mean±SD. (n=3)

Figure 4.1 Physical appearances of niosomes formulations of PR-niosomes in difference ratio between surfactant and cholesterol at 1 mmol of total content.

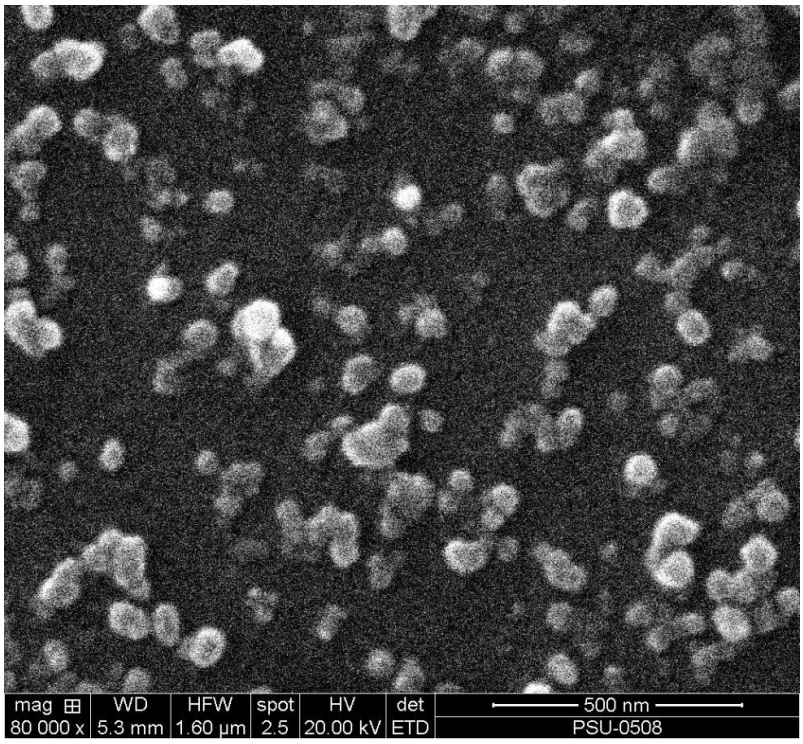


Figure 4.2 The morphology of niosomes formulation no. 9 investigated by SEM used magnification at 80,000.

The optimized PR-niosomes was chosen with the criteria as particle size lower than 500 nm with entrapment efficiency more than 50%. In this study, the formulation no. 9 was chosen. The formulation no. 9 has properties as 413±121.65 nm in particle size and 73.50±0.04% of entrapment efficiency, this formulation was chosen for further stability testing.

4.2 Stability of PR-niosomes

PR-niosomes formulation no. 9 was used for studied stability testing by storage in a refrigerator (4°C) and room temperature for 2 months. In this study, freshly formulation did not show precipitation, and it had yellow to orange in color.

After storage at 4°C for 4 weeks, the formulation exhibited the conglomeration of drug at the bottom of the glass container as showed in Figure 4.3 (b), which it represent unstable of PR-niosomes formulation. However, the precipitation did not occur after storage at room temperature for 4 weeks. The formulation at room temperature was continuously studied and it occurred the conglomeration of drug at 6 weeks of storage. The formulation with precipitation at 4°C was excluded from the studied, and then the formulation at room temperature was continuously studied and investigated. The stability of niosomes was studied the physicochemical properties of formulation such as entrapment efficiency, particle size, zeta potential and pH as shown in Table 4.3.

The stability of PR-niosomes in term of particle size and zeta potential was shown in Table 4.3. The result showed particle size of PR-niosomes changed after

storage both temperature. At 4°C, PR-niosomes changed from the beginning (399.3 nm) to 240.5 nm in 4 weeks, and it changed to 279.5 nm at room temperature in 6 weeks. The changing of particle size also occurred in the many studies such as the stability of ellagic acid loaded niosomes which it was investigated the mean size vesicle and found the change in size but not significant (Junyaprasert *et. al*., 2012). As same as the result of insulin-loaded niosomes by using Brij 72 as surfactant, the size had changed but not significant after storage at 4°C (Pardakhthy *et. al*., 2007).

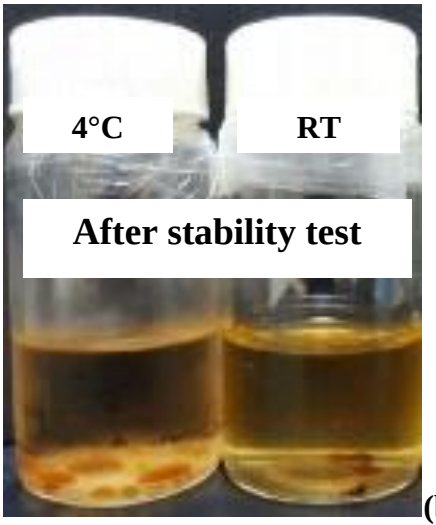
In addition, zeta potential of PR-niosomes also investigated and found that had not significant when stored at both temperatures. The zeta potential represent the repulsion between vesicle of niosomes, the stable of niosomes should have zeta potential value at ±30 mV or higher than (Sezgin-Bayindir *et. al*., 2014). The negative zeta potential of vesicle due to the hydroxyl group in cholesterol molecule which it occurs from the oxygen molecule gave partial negative property and hydrogen molecule gave partial positive property (Manosroi *et. al*., 2010). The zeta potential value of PR-niosomes had changed from -31.95 to -27.80 mV after stored at 4°C for 4 weeks, and changed to -25.50 mV after storage at room temperature for 6 weeks. From the result, the formulation showed low electrostatic repulsion of the vesicle, then it showed the short stability of niosomes. Like the result of Candesartan Cilexetil loaded in niosomes formulations, the formulation with a value lower than -30 mV of zeta potential had aggregation or sedimentation after storage at 5 and 25°C within 6 hours and 24 hours, respectively (Sezgin-Bayindir *et. al*., 2014).

The entrapment efficiency of PR-niosomes from stability testing at both temperatures were significantly decreased after storage. The entrapment efficiency of

PR-niosomes stored at 4°C for 4 weeks had decreased from 89.61% to 52.60% and decreased from 89.61% to 51.08% when storage at room temperature for 6 weeks. From the result, it showed leakage of PR from vesicle to medium which it was observed at the bottom of glass containers. Parthibarajan *et. al*. (2013) reported the result of the entrapment efficiency of voriconazole niosomes which had slightly decreased after storage for one month and the degradation of lipid bilayer results to leakage of drug from vesicles. The result of Junyaprasert *et. al*. (2013) reported percent of EA remaining in niosomes formulation after stability testing had decreased around 40-60%, and they explained about the reveal of type of surfactant, molarity ratio of surfactant and cholesterol affect to stability of niosomes, and the improvement of phase transition temperature will be increased stability of niosomes too.

The change of pH value was slightly increased in the stability testing. It showed slightly increased from 4.68 to 4.78 when stored at 4°C for 4 weeks, and 4.85 when storage at room temperature for 6 weeks. According to the result of pH value from gallidermin (Gdm) niosomes stability testing, it was found pH value slightly increased from initial. This phenomenon was described from the hydrolysis of the acyl chain of surfactant was reduced because surfactant contains ether linkage between a polar head group and the acyl group of surfactant (Manosroi *et. al*., 2010).

(a)



(b)

Figure 4.3 Physical appearances of PR-niosomes formulation (a) before and (b) after of stability testing at 4°C and room temperature (RT).

Table 4.2 Physicochemical properties of PR-niosomes formulation (No. 9) before and after stability testing.

	Before stability testing	**After stability testing**	
		4±2°C	**30±2°C**
Entrapment efficiency (%)	89.61 ± 0.05	52.60±0.11	51.08±0.12
Particle size (nm)	399.3 ± 17.26	240.5 ± 14.51	279.5 ± 4.68
Zeta potential (mV)	-31.95 ± 3.33	-27.80 ± 3.97	-25.50 ± 3.39
PDI value	0.900 ± 0.03	0.289 ± 0.04	0.344 ± 0.12
pH value	4.68 ± 0.04	4.78 ± 0.02	4.85 ± 0.02

*Results were represented as mean±SD. (n=3).

4.3 *In vitro* skin permeation studies

In this study, PR-niosomes formulation was investigated compared with PR-solution at the same concentration (25 mg/ml). Figure 4.4 showed cumulative amount of PR permeated through the skin for 24 hours. After studied for 24 hours, the cumulative amount of PR from PR-niosomes (16.49 ± 1.14 %) showed significantly higher when compared with PR-solution (3.35 ± 0.33 %) ($p<0.05$) as shown in Table 4.3. This agreed with the result of enoxacin niosomes gave a higher amount of drug than free drug after studied permeation of skin, the result was explained by the property of surfactant which acts as skin enhancer then it will increase the ability of drug permeated through the skin (Fang *et. al.*, 2001). It effects to the accumulation amount of PR in the skin which was investigated at the end of the study.

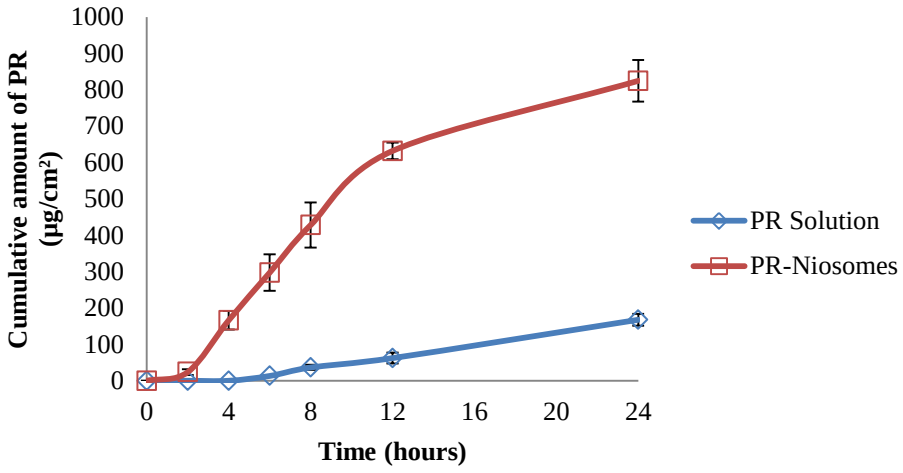


Figure 4.4 *In vitro* cumulative amount of PR vs. time profile permeated across pig skin from PR-Solution and PR-Niosomes formulation for 24 hours.

Table 4.3 *In vitro* skin permeation parameters of PR from solution and niosomes formulation after 24 hours (n=3).

Formulations	Q_{cum} (%)	J_{ss} (µg/cm²/h)	K_p (* 10^{-3} cm²/h)
Solution	3.35 ± 0.33	6.98 ± 0.69	1.40 ± 0.14
Niosomes	16.49 ± 1.14	34.35 ± 2.37	6.87 ± 0.47

The in vitro release data was used to predict the drug release kinetic mechanism by applying to various kinetic models such as Zero order model, First order model, and Higuchi's model. The data in Table 4.3 showed the calculated results from a slope of each plot, and regression coefficient (R^2) also determined. The release data of PR-niosomes followed Higuchi's model of release that it mean drug was released from matrix system (Dash *et. al*., 2010).

Table 4.4 Release kinetic of PR-niosomes formulation.

Sample	Zero-order model (R^2)	First-order model (R^2)	Higuchi model (R^2)
PR-Niosomes	0.8886	0.5498	0.9669

The amount of PR accumulated in skin after study for 24 hours was expressed in Figure 4.5, PR-solution was higher accumulated in pig skin than PR-niosomes formulation (162.82 ± 0.93 and 132.40 ± 2.93 µg/cm², respectively). This might be the property of surfactant in niosomes formulation increased the permeation of drug across the skin than solution, and then PR from solution was found in skin more than niosomes.

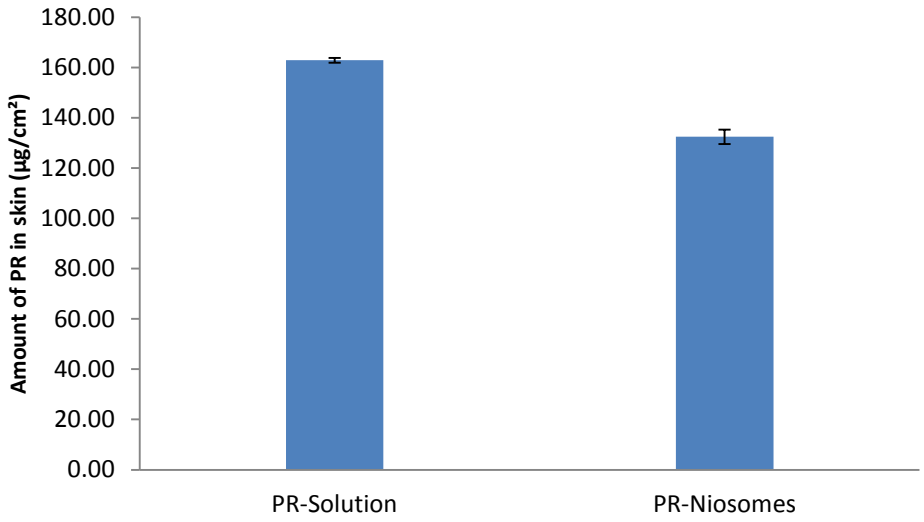


Figure 4.5 The cumulative amount of PR in skin from PR-solution and PR-niosomes formulation. The result represent as mean±SD (n=5).

4.4 Skin irritation test

The selected formulation of PR-niosomes (no.9) was studied the skin irritation on rabbit skin, the studied was performed by Thailand Institute of Scientific and Technological Research (TISTR). Distilled water was used to compare with PR-niosomes.

After investigated reactions at 1, 24, 48, and 72 hours. The rabbit skin did not exhibit the dermal irritation. The scores of dermal reaction were shown in Table 4.4. Although, PR-niosomes permeated across skin to lower systemic but it was not found irritation when it was applied on skin.

Table 4.5 The scores of dermal reaction on the skin of rabbits.

Scoring time (hour)		Formulations	
		Distilled water	**PR-Niosomes**
1	**Erythema**	0	0
	Oedema	0	0
24	**Erythema**	0	0
	Oedema	0	0
48	**Erythema**	0	0
	Oedema	0	0
72	**Erythema**	0	0
	Oedema	0	0

4.5 Cream base formulation

Cream base formulations were prepared and accelerating tested by freeze/thaw method for 5 cycles, The physical appearance, viscosity and pH value were investigated as shown in Table 4.5. The physical appearance, color and texture were shown in Figure 4.6. After accelerating test, all formulations were investigated physical appearance and compared with the data before testing. However, all of formulations had minor changes of pH value and viscosity, then PR-solution and PR-niosomes were incorporated with all cream base formulations.

Table 4.6 Viscosity and pH value of cream base formulations, before and after accelerating test by freeze/thaw for 5 cycles.

Formulation no.	Before testing		After testing	
	pH value	**Viscosity (x10^3 cP)**	**pH value**	**Viscosity (10^3 cP)**
1	5.24 ± 0.04	139.83 ± 4.07	5.31 ± 0.01	147.36 ± 7.17
2	5.21 ± 0.05	116.04 ± 3.78	5.19 ± 0.01	113.93 ± 6.25
3	5.04 ± 0.01	76.32 ± 3.97	5.01 ± 0.01	74.38 ± 5.21

Data represent as mean±SD. (n=3)

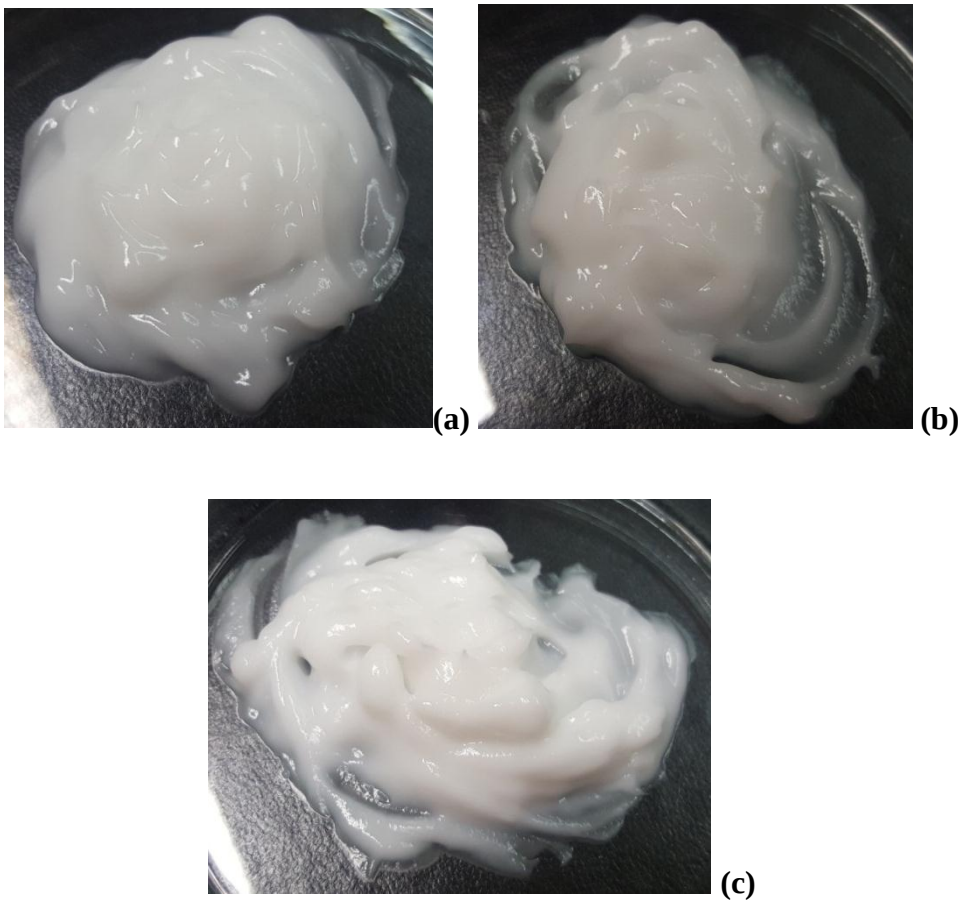


Figure 4.6 Cream base formulations after freeze/thaw 5 cycles; (a) Formulation no. 1; (b) Formulation no. 2; (c) Formulation no. 3.

When the active ingredient was incoperated to cream base formulations, it was studied accelerating test by freeze/thaw method for 5 cycles and was investigated physical appearance, pH value, and viscosity. Figure 4.7 and Table 4.6 showed the physical appearance, pH value and viscosity of all formulations. At the end of study, the data was compared with before testing, and formulation no. 2 was selected to study for long term stability which it showed good physical appearance, minor changed in viscosity, and had pH value closely to pH of skin, 4.5-5.5.

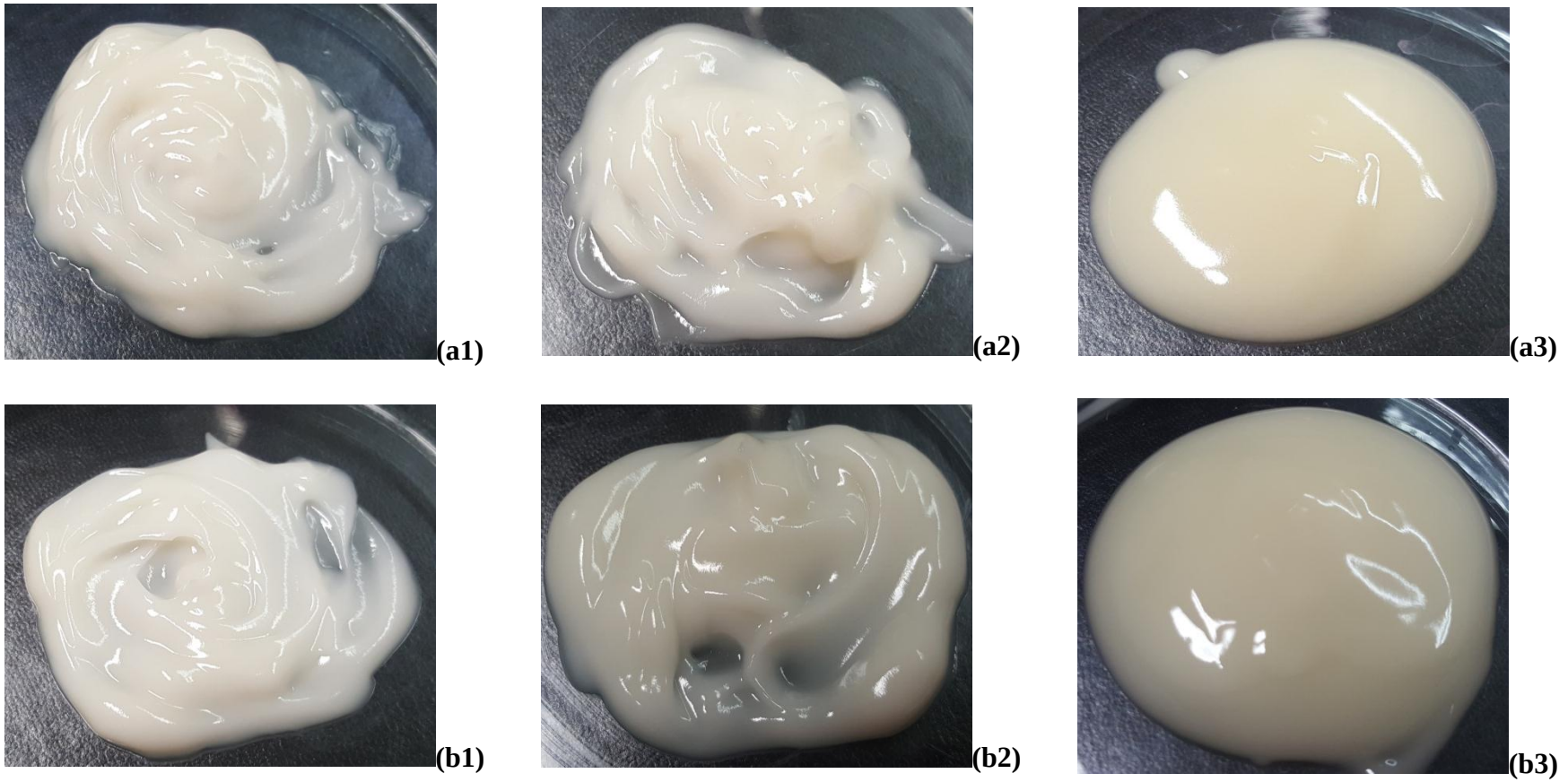


Figure 4.7 Physical appearance of PR cream(a) and PR-niosomes cream (b) after freeze/thaw 5 cycles.

Table 4.7 Viscosity and pH value of PR cream and PR-niosomes cream, before and after accelerating test by freeze/thaw for 5 cycles.

Formulation no.		PR-solution cream		PR-niosomes cream	
		pH value	**Viscosity ($x10^3$ cP)**	**pH value**	**Viscosity ($x10^3$ cP)**
1	**Before**	5.61 ± 0.01	76.19 ± 1.30	5.54 ± 0.03	69.84 ± 3.25
	After	5.16 ± 0.04	67.28 ± 1.59	5.52 ± 0.01	66.94 ±2.64
2	**Before**	5.88 ± 0.01	50.36 ± 0.54	5.87 ± 0.03	47.01 ± 2.86
	After	5.57 ± 0.01	49.18 ± 3.71	5.85 ± 0.01	45.30 ± 2.28
3	**Before**	6.52 ± 0.02	16.06 ± 1.73	6.56 ± 0.03	14.46 ± 1.03
	After	6.54 ± 0.01	15.49 ± 0.98	6.51 ± 0.01	14.79 ± 1.21

Data represent as mean±SD. (n=3)

4.6 Stability of cream contained PR-niosomes

The stability of PR-niosomes cream was storaged at three different temperature (4°C, room temperature, and 45 °C) for two months, and was investigated total drug content (%), pH value, viscosity, and physical appearance (Figure 4.8). PR-niosomes cream was studied and compared with PR-solution cream at the same conditions.

Table 4.7 shows the comparation before and after stability test, it was investigated total drug content, pH value and viscosity of formulation. The result found the change of pH value in PR-cream may effect to viscosity and total drug content which it effect to decrease viscosity of formulation and also decrease total drug content. In PR-niosomes formulation, the change of pH value had minor effect to viscosity and total drug content of formulation, however the viscosity of formulation also decreaed but it less than the viscosity of PR cream. At the end of test, total drug content of PR-niosomes formulation showed higher than data of PR cream, this may effect to protect drug from oxidation process of niosomes.

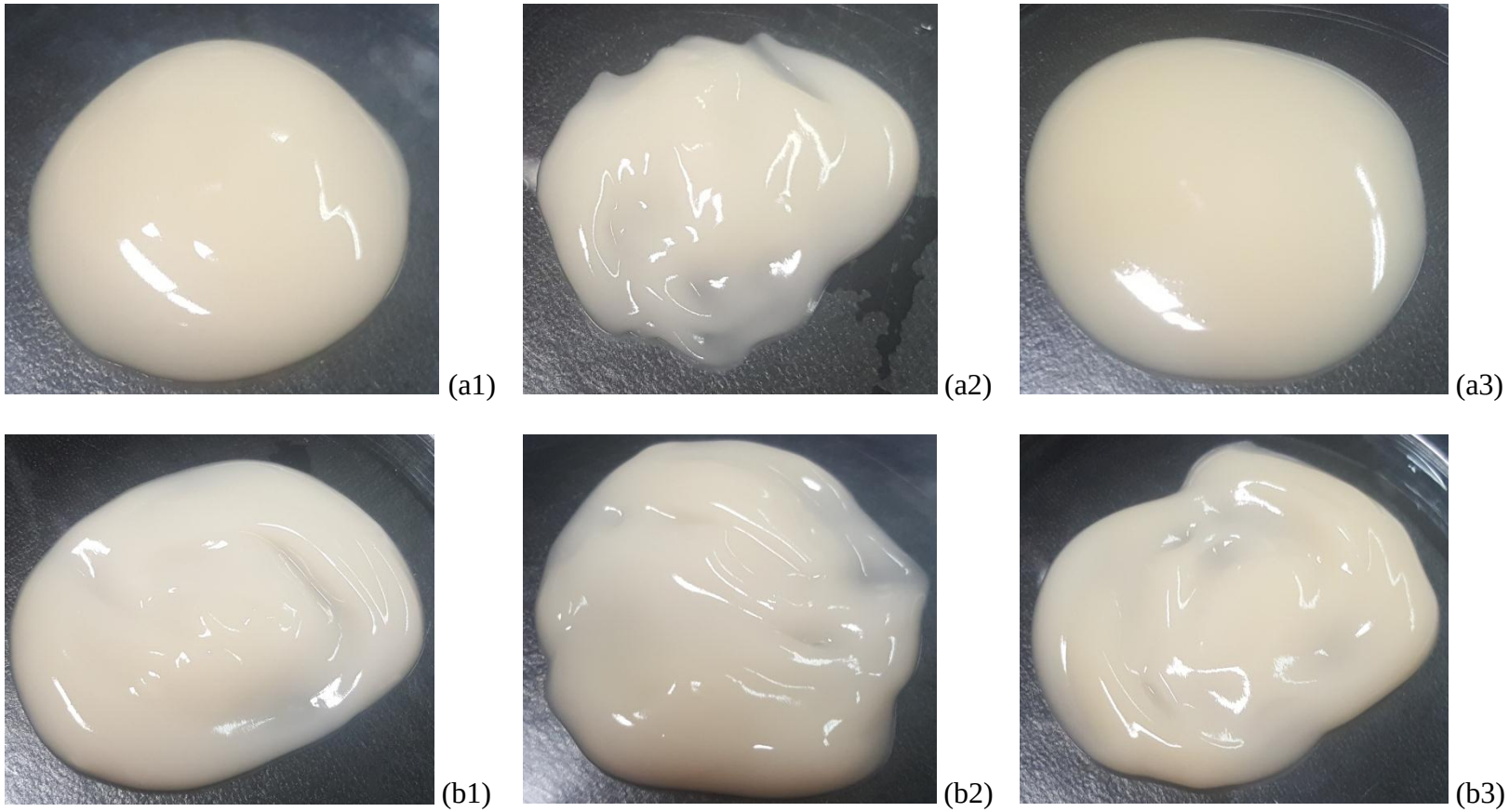


Figure 4.8 Physical appearance of PR cream (a) and PR-niosomes cream (b) at difference storage temperature 4°C (1), room temperature (2) and 45 °C (3).

Table 4.8 Viscosity, pH value and total drug content of PR cream and PR-niosomes cream compared between before and after storage for 2 months at three temperatures.

			Formulations	
			PR-cream	**PR-niosomes cream**
Viscosity (x 10^{-3} cP)	**Before testing**		24.24 ± 0.57	28.99 ± 0.94
	After storage for 2 months	**4°C**	16.75 ± 0.57	23.31 ± 0.47
		Room temperature	15.49 ± 0.11	24.43 ± 0.47
		45°C	15.68 ± 0.29	24.49 ± 0.22
pH value	**Before testing**		5.61 ± 0.03	5.52 ± 0.01
	After storage for 2 months	**4°C**	5.68 ± 0.01	5.45 ± 0.00
		Room temperature	5.66 ± 0.01	5.43 ± 0.00
		45°C	5.72 ± 0.01	5.39 ± 0.00
Total active content (%)	**Before testing**		99.11 ± 2.54	94.97 ± 0.45
	After storage for 2 months	**4°C**	61.96 ± 0.71	73.97 ± 2.08
		Room temperature	64.18 ± 1.27	73.62 ± 0.93
		45°C	61.76 ± 0.28	70.79 ± 0.28

Data were expressed as mean±SD (n=3).

4.7 *In vitro* skin permeation study of cosmetic formulation contained PR-niosomes

In vitro skin permeation study of cosmetic formulation contained PR-niosomes was studied and compared with PR-Solution cream at the same concentration (0.5% of PR w/w). At the end of study (24 hours), the amount of PR accumulated in pig skin was investigated and the result was performed in Figure 4.9.

The amount of PR from niosomes cream (123.32 ± 2.18 µg/g) deposited on skin showed significantly higher than PR from solution cream (56.83 ± 2.25 µg/g) (p<0.05). The amount of PR was not found in receptor compartment in both of formulations, this phenomenon can be explained by the property of cream formulation act as reservoir of drug as same as the result of *in vitro* releases of Gdm loaded in niosomes and incorporated with gel formulation, which no Gdm found in receptor medium (Manosroi *et. al.*, 2010). This effect to low risk of systemic irritation from PR-niosomes. From the previous study of permeation was found the amount of PR from solution deposited in skin higher than niosomes formulation, however in the result of this study was found inversely. This phenomenon may be from the property of some ingredient in cream formulation, propylene glycol, which able to increase or decrease penetration of drug. The study of Wagner *et. al.* (2004) showed the result of the addition propylene glycol into polyacrylate gel formulation it reduced penetration for lipophilic drug.

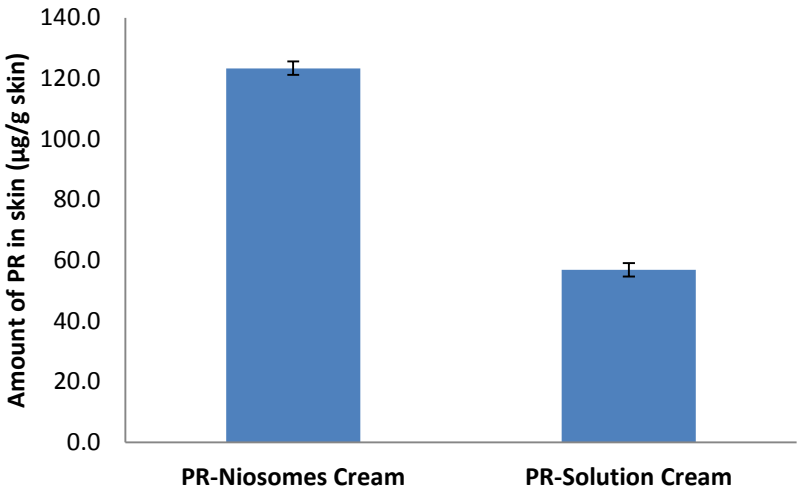


Figure 4.9 The cumulative amount of PR in skin from PR-Niosomes cream and PR-solution cream. The result represent as mean±SD (n=5).

CHAPTER 5

CONCLUSION

From this study, niosomes was developed to entrap phenylethyl resorcinol (PR) for increase efficiency of PR when using with cosmetic formulation. The advantages of niosomes had interested for our studied.

Brij™72 was used in this study and it was cooperated with cholesterol to develop the entrapment efficiency of niosomes. For this experiment, twelve formulations was performed to investigate and evaluated in physicochemical properties such as vesicle size, zeta potential, polydispersity index (PDI) and entrapment efficiency. Firstly, the effect of compositions between surfactant and cholesterol were investigated at difference ratio at 1 mmol of total content, it found the suitable ratio was 1:1 of surfactant and cholesterol. The formulation no. 6 showed the highest entrapment efficiency as 55.07 ± 0.20%, 362.03 ± 35.23 nm of vesicle size with 0.62 ± 0.26 of PDI value and -33.20 ± 1.75 mV of zeta potential. To improve entrapment efficiency, increasing of total content of Brij™72 and cholesterol was performed which varied at 1.2, 1.4, 1.6, 1.8 and 2.0 mmol. Physicochemical properties also investigated as same as previously, the results found the good formulation which included our criteria is formulation no. 9 which it had 413 ±

121.65 nm in size and 73.50 ± 0.04% of entrapment efficiency. Then, this formulation was studied stability test and irritation test, it gave 6 weeks of stability and no irritation reaction on rabbit skin.

The selected formulation was incorporated with cream base and evaluated for *in vitro* skin permeation study compared with PR cream. The *in vitro* skin permeation study of PR-niosomes was found significant higher of parameter (Q_{cum}: 16.49±1.14%, J_{ss}: 34.35±2.37 µg/cm²/h and K_p: 6.87±0.47 $*10^{-3}$ cm^2/h) than PR solution. The accumulation of PR in skin showed higher amount from PR solution when compared with PR-niosomes, this phenomenon explained by the property of surfactant which it may destroyed structure of skin and lead to permeation of PR. However, the accumulation of PR in skin of PR-niosomes formulation performed higher than PR cream (123.32±2.18 and 56.83±2.25 µg/g, respectively), the result showed effect of niosomes could increase the accumulation of PR in skin.

From the study showed the potential of niosomes to improve the using of PR in cosmetic formulation in term of product development and skin carrier. In addition, it also safety with skin application using niosomes incorporated with topical formulation.

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APPENDIX

1. Quantitative analysis of PR using HPLC

1.1 Materials and methods

1.1.1 Standard solution

Standard solution was prepared by dissolving PR 50.0 mg by methanol 50 ml, the result was stock solution at concentration is 1000 µg /ml. Stock solution will be stored at 4°C, freshly preparation should be done. Methanol was used for dilute the stock solution.

1.1.2 Instrument and chromatographic conditions

The analytical was adapted from method of Fan *et al*. (2014) for appropriate in this experiment. The condition of chromatographic analysis was done with a reverse phase BDS HYPERSIL C_{18} column (150 × 4.6 mm, particle size 5 µm) at room temperature. The mobile phase was mixture of acetonitrile/ methanol/ milli-Q water (40:20:40 v/v) which was filtered through 0.45 µm nylon membrane filter and degassed by sonication before using. The flow rate was 0.8 ml/min. The injection volume of sample was 20 µl and was detected at 254 nm. The analysis of PR was performed by HPLC Agilent 1100 series (Waldbronn, Germany).

1.1.3 The validation procedures

Specificity

Determination of material will be express by chromatogram from HPLC analysis. Chromatogram will be compare between peak area of PR and peak of mixture, the peak of PR should be separately from the other peaks.

Linearity and range

The standard solutions were prepared in range of 5-50 µg/ml of PR concentration for analysis of and 1-20 µg/ml for analysis PR in pig skin. Calibration curve was constructed from the plot between concentration of standard solution (X-axis) and peak area of chromatogram (Y-axis). Linearity was expressed by linear regression equation. Correlation coefficient (R^2) was used for the reliable degree of linearity in the experiment, it should be greater than 0.999.

Limit of detection and Limit of quantification

Both of LOD and LOQ will be calculated by standard deviation and slope of calibration curve as following equation;

$$LOD = 3.3\sigma / S$$

$$LOQ = 10\sigma / S$$

Where σ is the standard deviation of response

S is slope of calibration curve

Accuracy and precision

Accuracy and precision for analytical of PR content in niosomes formulations were performed by spiking the PR standard solution in blank niosomes at 18.75, 25 and 31.25 µg/ml and 7.5, 10 and 12.5 µg/ml in blank skin. These are low, medium and high of PR concentration in the test. Samples were extracted by methanol in the same volume and dilute before analyze with HPLC. The PR samples were freshly prepared and analyzed in triplicated for each concentration to determine the intra-day accuracy and precision. The same procedure was done for three different days for determining the inter-day accuracy and precision.

For accuracy, percent recovery of PR was calculated from the linear regression equation and were compared with true value which can be calculated by the following equation;

$$\%\ Recovery = \frac{C_{Measured}}{C_{Actual}} \times 100$$

Where $C_{Measured}$: Concentration of PR detected by HPLC

C_{Actual} : Actual concentration of PR

For precision, all of precision methods will be calculate standard deviation and percent relative standard deviation (%RSD) which should not more than 2.0%. The following equation will be used for calculate percent relative standard deviation;

$$\%RSD = (SD / \bar{X}) \times 100$$

1.2 Results and discussion

1.2.1 Quantitative determination of PR using HPLC

A typical chromatogram of standard solution of PR was shown in Figure 6.1. The retention time of PR was about 4.5 minute.

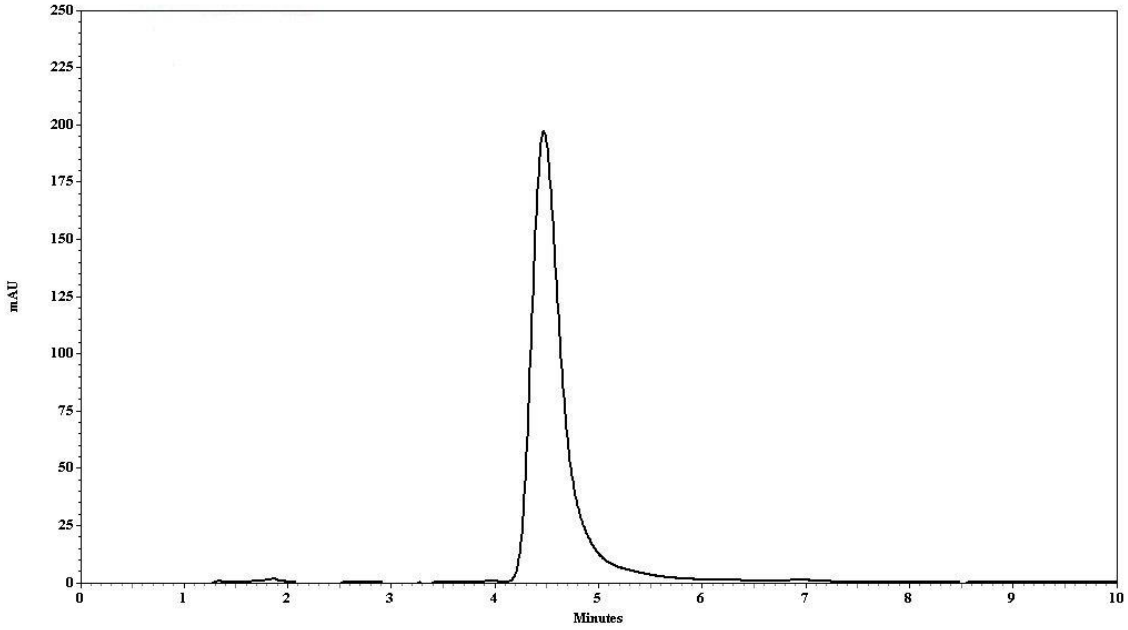

Figure 6.1 Chromatogram of standard solution of PR.

1.2.2 Validation of analytical method

Specificity

The analytical method was performed for determination PR in niosomes, cream formulation and *in vitro* skin permeation studies. The peak of PR should be able to identify from all of formulation of test. Figure 6.2, 6.3, 6.4 and 6.5 expressed

the specificity of the chromatogram from the analysis of PR in receptor compartment, membrane pig skin and cream base, respectively.

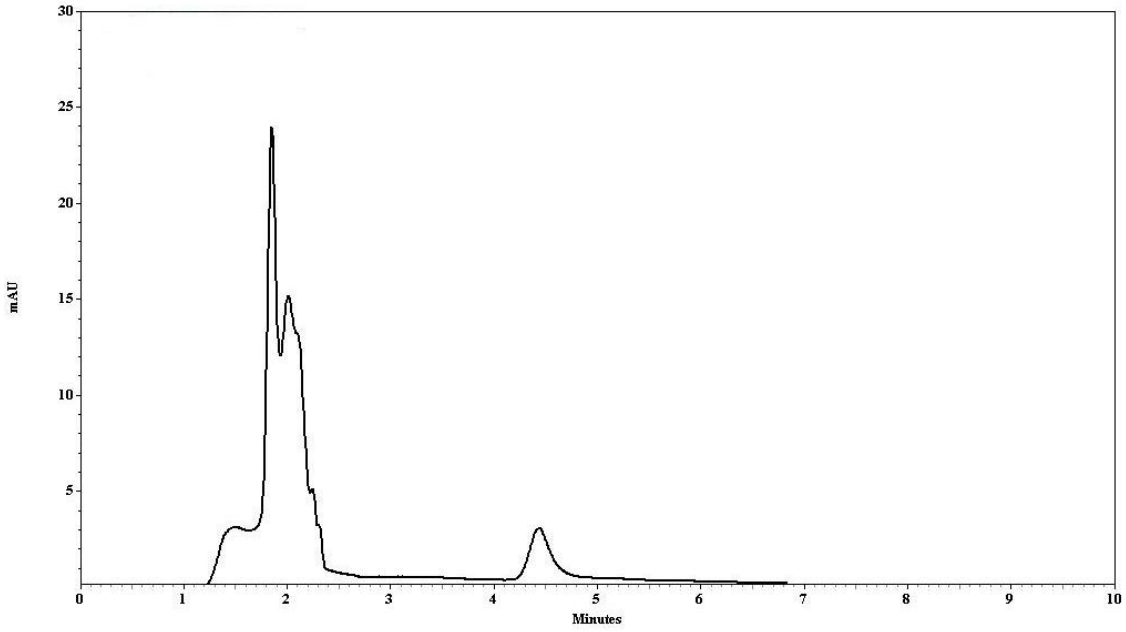

Figure 6.2 Chromatogram of PR spiked in receptor fluid.

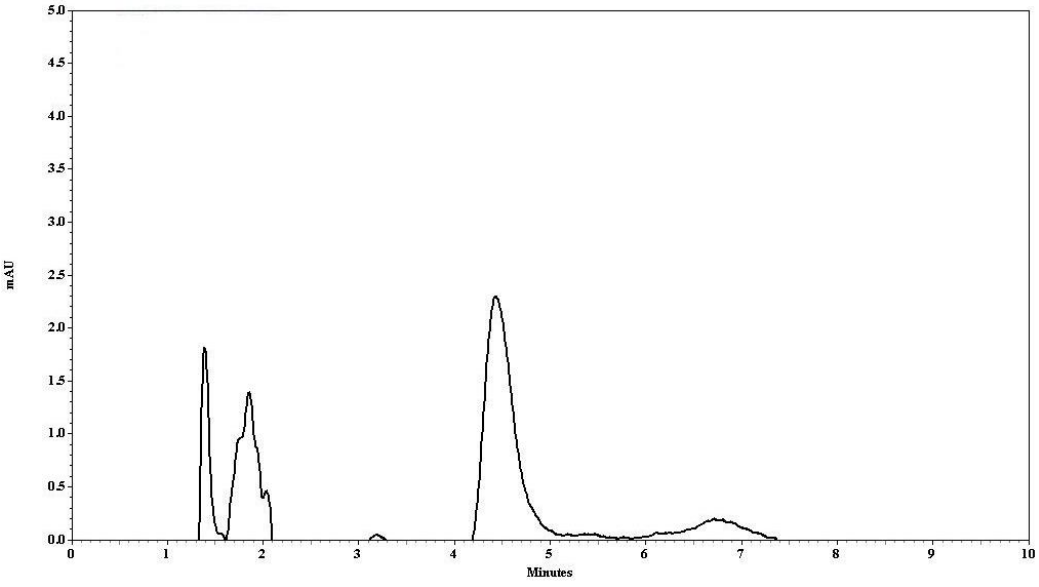

Figure 6.3 Chromatogram of PR spiked after extracted by methanol.

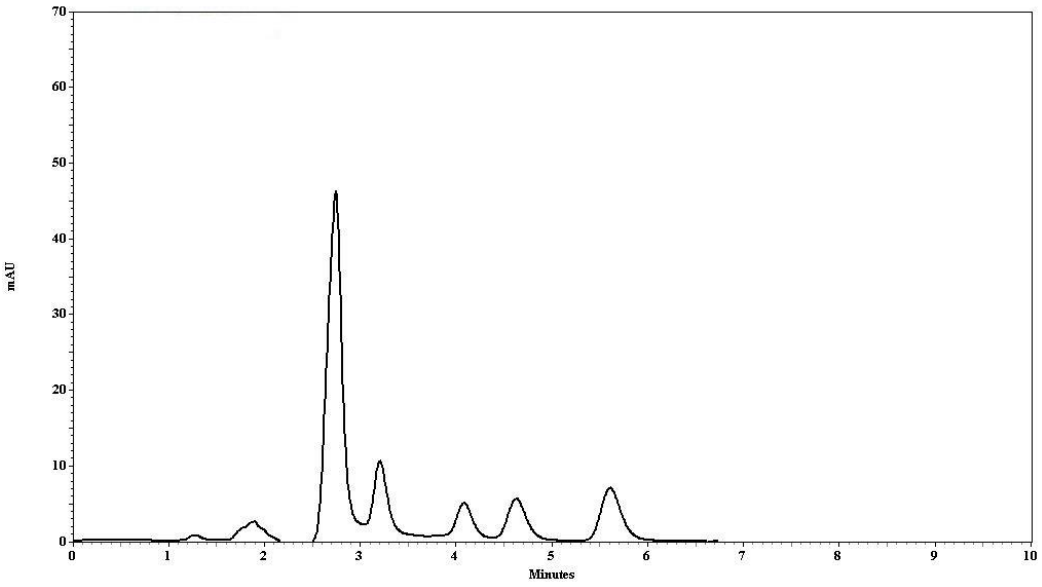

Figure 6.4 Chromatogram of PR spiked in cream base.

Linearity

The standard solutions were prepared in range of 5-50 µg/ml of PR concentration for analysis of PR and 1-20 µg/ml for analysis PR in pig skin. Calibration curve was constructed from the plot between concentration of standard solution (X-axis) and peak area of chromatogram (Y-axis). Linearity was expressed by linear regression equation. Correlation coefficient (R^2) was used for the reliable degree of linearity in the experiment, it should be greater than 0.999.

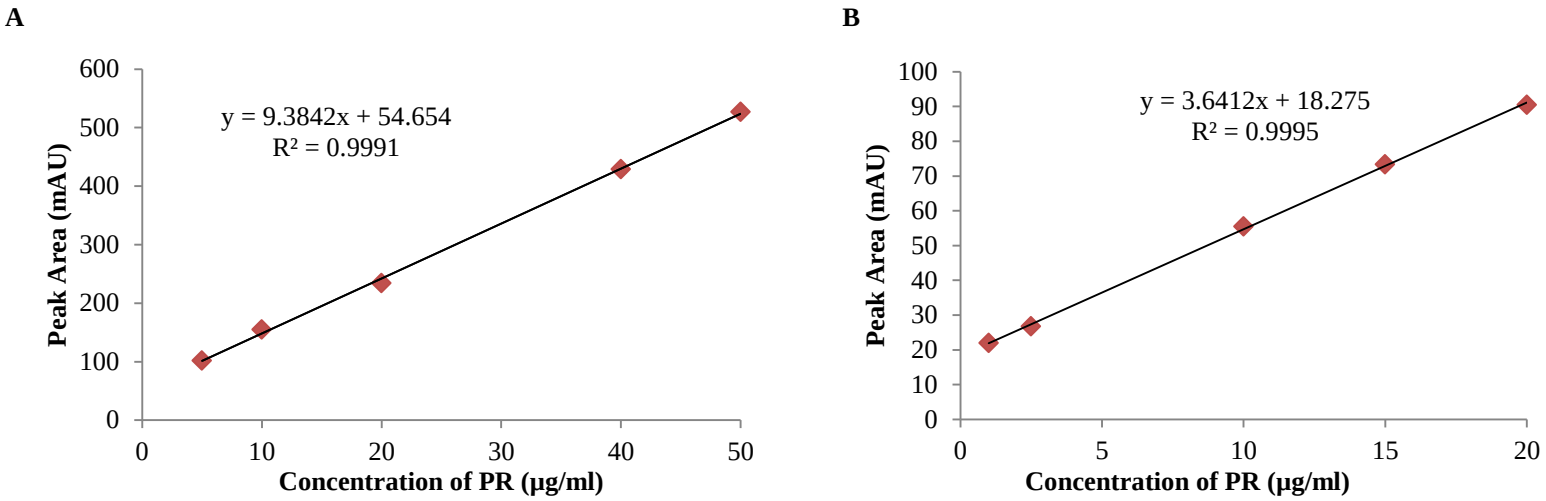

Figure 6.5 Standard calibration curve of PR for analysis PR in niosomes formulation and cream (A), in membrane pig skin (B). The plotted data expressed as mean±SD (n=3).

Limit of detection and Limit of quantification

By calculating based on the response and slope, it was found that the LOD and LOQ value for PR were 0.43 and 1.29 µg/ml, respectively.

Accuracy and precision

The accuracy and precision were evaluated from intra-day and inter-day of the assay. Table 1 shows the intra-day reproducibility of PR and in pig skin. At each concentration, the intra-day RSD varied between 0.07-1.40 % in the analysis of PR and 0.35-1.35 % in the analysis of PR in pig skin. The accuracy of the average measured concentration for each day was reported as % recovery varied between 74.35-94.52% for the analysis of PR and 88.46-99.77% for the analysis of PR in pig skin. Table 2 shows the inter-day analysis which RSD varied between 1.08-1.93% for the analysis of PR and 1.36-1.99% for the analysis of PR in pig skin. The percent recovery was expressed 78.62-88.72% and 89.64-97.60% for the analysis of PR and in pig skin, respectively.

Table 1 Intra-day variable of PR analysis.

Spiked conc. (µg/ml)	Day	Mean of measured conc. (µg/ml)	Average recovery (%)	% RSD
The analysis of PR				
18.75	1	16.60 ± 0.04	88.55	0.27
	2	15.39 ± 0.04	82.09	0.25
	3	16.33 ± 0.23	87.08	1.40
25.00	1	21.10 ± 0.04	84.40	0.20
	2	23.11 ± 0.11	92.43	0.47
	3	21.24 ± 0.28	84.94	1.33
31.25	1	23.32 ± 0.02	74.61	0.07
	2	29.54 ± 0.09	94.52	0.31
	3	23.20 ± 0.22	74.35	0.96
The analysis of PR in pig skin				
7.50	1	6.63 ± 0.09	88.46	1.33
	2	6.82 ± 0.08	90.90	1.21
	3	6.72 ± 0.09	89.56	0.62
10.0	1	9.98 ± 0.12	99.77	1.21
	2	9.70 ± 0.10	97.01	1.02
	3	9.60 ± 0.13	96.01	1.35
12.5	1	11.92 ± 0.04	95.33	0.35
	2	11.72 ± 0.10	93.72	0.82
	3	11.47 ± 0.11	91.75	0.97

The data expressed as mean ± SD (n=3).

Table 2 Inter-day variable of PR analysis

Spiked conc. (µg/ml)	Mean of measured conc. (µg/ml)	Average recovery (%)	% RSD
The analysis of PR			
18.75	16.63 ± 0.32	88.72	1.93
25.00	21.30 ± 0.23	85.19	1.10
31.25	24.57 ± 0.27	78.62	1.08
The analysis of PR in pig skin			
7.50	6.72 ± 0.09	89.64	1.36
10.00	9.76 ± 0.19	97.60	1.99
12.50	11.70 ± 0.22	93.60	1.92

The data expressed as mean ± SD (n=3).

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